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Impacts of Gaseous Ozone (O₃) on Germination, Mycelial Growth, and Aflatoxin B₁ Production In Vitro and In Situ Contamination of Stored Pistachio Nuts

Alaa Baazeem ¹, Angel Medina ² and Naresh Magan ^{2,*}

¹ Department of Biology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia; aabaazeem@tu.edu.sa

² Applied Mycology Group, Environment and AgriFood Theme, Cranfield University, Cranfield MK43 0AL, UK; a.medinavaya@cranfield.ac.uk

* Correspondence: n.magan@cranfield.ac.uk

Abstract: Pistachio nuts can become colonized by mycotoxigenic fungi, especially *Aspergillus flavus*, resulting in contamination with aflatoxins (AFs). We examined the effect of gaseous O₃ (50–200 ppm; 30 min; 6 L/min) on (a) in vitro germination, (b) mycelial growth, and (c) aflatoxin B₁ (AFB₁) production on a milled pistachio nut-based medium at different water activity (a_w) levels and at 30 °C. This was complimented with in situ studies exposing raw pistachio nuts to 50–200 ppm of O₃. Exposure of conidia to gaseous O₃ initially resulted in lower germination percentages at different a_w levels. However, 12 h after treatment, conidial viability recovered with 100% germination after 24–48 h. Growth rates of mycelial colonies were slightly decreased with the increase of the O₃ dose, with significant inhibition only at 0.98 a_w. The production of AFB₁ after O₃ treatment and storage for 10 days was stimulated in *A. flavus* colonies at 0.98 a_w. Raw pistachio nuts inoculated with *A. flavus* conidia prior to O₃ exposure showed a significant decrease in population after 20 days of storage. However, AFB₁ contamination was stimulated in most O₃ treatments. The relationship between exposure concentration, time and prevailing a_w levels on toxin control needs to be better understood for these nuts.

Keywords: gaseous ozone; germination; growth; aflatoxin B₁; water availability; *Aspergillus flavus*; pistachios

Key Contribution: This is the first detailed study of the potential for the use of gaseous ozone treatment to try and control different life-cycle phases of *A. flavus* on milled raw pistachio-based media and in situ control of *A. flavus* populations and aflatoxin B₁ in raw stored shelled pistachio nuts when treated and stored under different water activity conditions.



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1. Introduction

Ozone (O₃) is commonly generated in the summer because of the reactions between photochemicals in the atmosphere in the presence of sunlight. O₃ gas has been commonly used in the food industry for sanitizing packaging materials, raw materials, and storage facilities [1]. It has been suggested that the advantage of O₃ over other remedial chemicals used in foodstuffs is that it is residue-free. It decomposes to diatomic oxygen rapidly because of its short half-life, which is about 20–50 min in the atmosphere and 1–10 min in water [2]. However, it is a very corrosive gas, and for its use, it is critical that appropriate tubing (PTFE), steel, or glass systems are used. Manning and Teidemann [3] showed that small increases in ozone (O₃) concentrations (40–60 ppb) can influence the mycobiota of plant surfaces and perhaps the biosynthesis of toxins. Slight increases in exposure to gaseous O₃ as a pollutant was previously shown to change the phyllosphere mycobiota on conifer needles [4].

At the present time, the EU legislation established a maximum level of 12 µg/kg aflatoxin B₁ (AFB₁) in nuts, including pistachios used for processing, and 8 µg/kg AFB₁ for direct human consumption [5]. The AF contamination of pistachio nuts occurs either pre-harvest, because of early splitting of the shells and insect damage, or during subsequent drying and processing [6]. For example, in Iran, an analysis of 10,000+ samples of pistachio nuts showed AFB₁ in 36%, with almost 12% exceeding the maximum level in Iran (5 µg/kg) [7]. In Algeria, between 1998 and 2002, the highest AFB₁ in 523 samples was 113 µg/kg, with a mean range of 1 to 3.8 µg/kg [8]. In Tunisia, 76% of pistachio nuts were found to be contaminated with AFs during storage [9]. Delayed harvesting and processing and storage has been shown to significantly influence and often increase the AFB₁ contamination of pistachio nuts [6,10]. Thus, there has been interest in examining treatments such as gaseous O₃ for the mitigation of AFs in nuts, including pistachios [11–13].

Some previous studies have examined the efficiency of electrochemically-generated O₃ on the activity of aflatoxigenic fungi and aflatoxin (AFs) in different nuts, including peanuts and brazil nuts. However, there are less studies on pistachio nuts [11–13]. Mylona et al. [14] showed that gaseous O₃ (100–400 ppm) was not very effective against the microconidial germination of *Fusarium verticillioides* in vitro, but it was able to reduce fumonisin B₁ (FB₁) contamination in stored maize grain after treatment. Indeed, the initial inhibition of germination was followed by recovery, growth, and FB₁ production. However, Sultan et al. [12] found that gaseous O₃ was very effective in inhibiting the germination of conidia of *A. flavus*, but it had little efficacy in controlling the mycelial growth of *A. flavus* strains on peanut-based media, regardless of water activity (a_w). Studies have been carried out on the O₃ treatment of Brazil nuts and showed that exposure to this gas affected the growth of the mycobiota and decreased AFs [11]. They used three concentrations of O₃ (10, 14, and 31.5 mg/L) for five hours and found that this inhibited colonization by both *A. flavus* and *A. parasiticus* over a storage period of 180 days. However, at low concentrations of O₃, the fungi were still able to grow. Care is needed with nuts because the high concentration of lipids can interact with gaseous O₃, resulting in tainting and off-flavors [15]. The sensitivity of fungal species to O₃ exposure may vary and depends on the exposure period, concentration applied, and type of commodity. It can also be influenced by moisture content and spore morphology [16,17].

No previous studies have examined the effect of gaseous O₃ (0–200 ppm) as a control measure for germination, colonization, and AFB₁ in raw milled pistachio-based media or in stored raw pistachios. Thus, the aim of this study was to examine the use of gaseous O₃ to control (a) in vitro conidial germination, (b) in vitro mycelial growth and AFB₁ production, and (c) *A. flavus* populations on raw pistachio nuts when inoculated with conidia of this species and exposed to O₃ and the effects on subsequent AFB₁ contamination after 20 days of storage at different a_w levels.

2. Results

2.1. Effect of O₃ on Conidial Germination of *A. flavus* In Vitro on a Milled Pistachio-Based Medium

The mean germination of conidia of *A. flavus* was influenced by gaseous O₃ treatment, regardless of the a_w level used (Figure 1). However, all the O₃-treated conidia showed a recovery in germination capacity after 48 h, regardless of a_w level. The only exceptions were the samples treated with 200 ppm O₃ at 0.98 a_w and all O₃ doses at 0.93 a_w after 12 h.

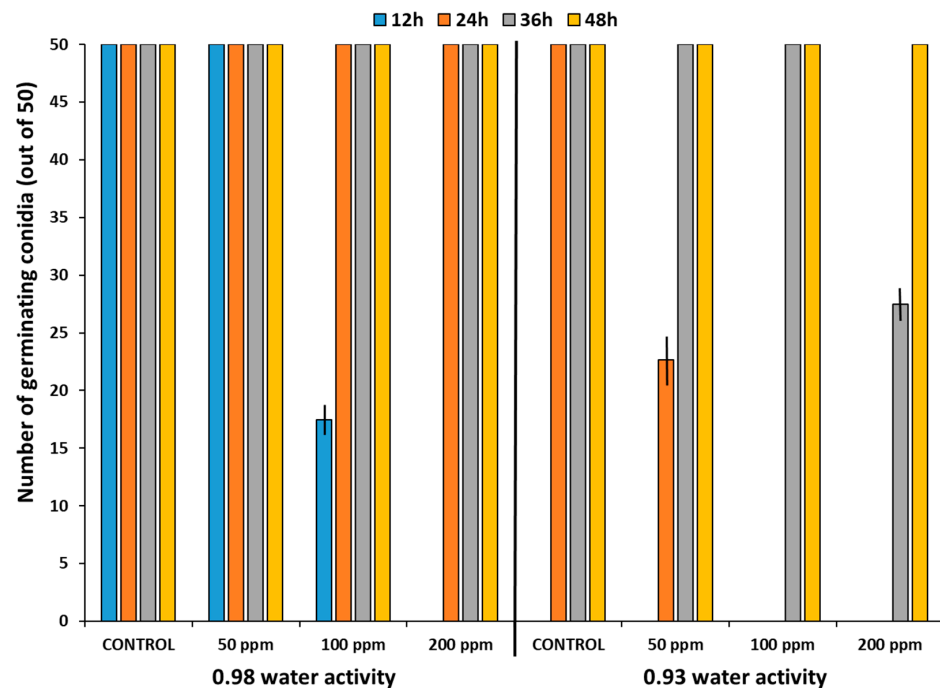


Figure 1. Effect of gaseous ozone (6 L/min for 30 min) on the mean number of conidia of *A. flavus* strain AB3 germinating (mean of 9×50 conidia) at 0.98 and 0.93 a_w on a 3% milled raw pistachio nut medium and then incubated at 30 °C. Bars indicate the SEM number of spores germinated.

2.2. In Vitro Effect of Gaseous O_3 Treatment on Mycelial Growth

The mycelial extension of *A. flavus* (strain AB3) was significantly inhibited after O_3 treatment when compared with the control at 0.98 a_w (Figure 2). However, the growth rates with the different O_3 exposure concentrations were relatively similar. At 0.93 a_w , growth rates were slightly higher after O_3 treatment, with little difference between the concentrations of O_3 used.

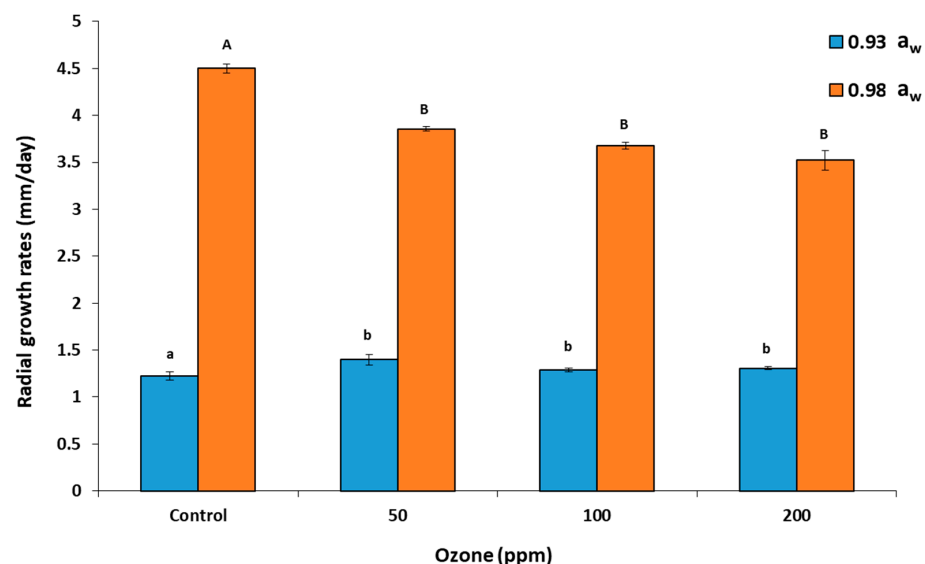


Figure 2. Effect of gaseous ozone treatment on the mycelial colony extension rate of *A. flavus* (strain AB3) on a 3% milled raw pistachio nut-based medium at 0.98 and 0.93 water activity (a_w) and 30 °C for 4–6 days. Colonies were exposed to gaseous ozone for 30 min at 6 L/min prior to incubation. Different letters indicate significant differences ($p < 0.05$). Bars indicate mean SE.

2.3. In Vitro Effect of Gaseous O₃ Exposure of Colonies of *A. flavus* on AFB₁ Production

The AFB₁ productions were significantly higher after O₃ treatments at all exposure levels when compared to the untreated control in the 0.98 a_w treatments (Figure 3). However, at 0.93 a_w, there was no significant effect on toxin production, with much lower AFB₁ productions on the raw pistachio-based medium.

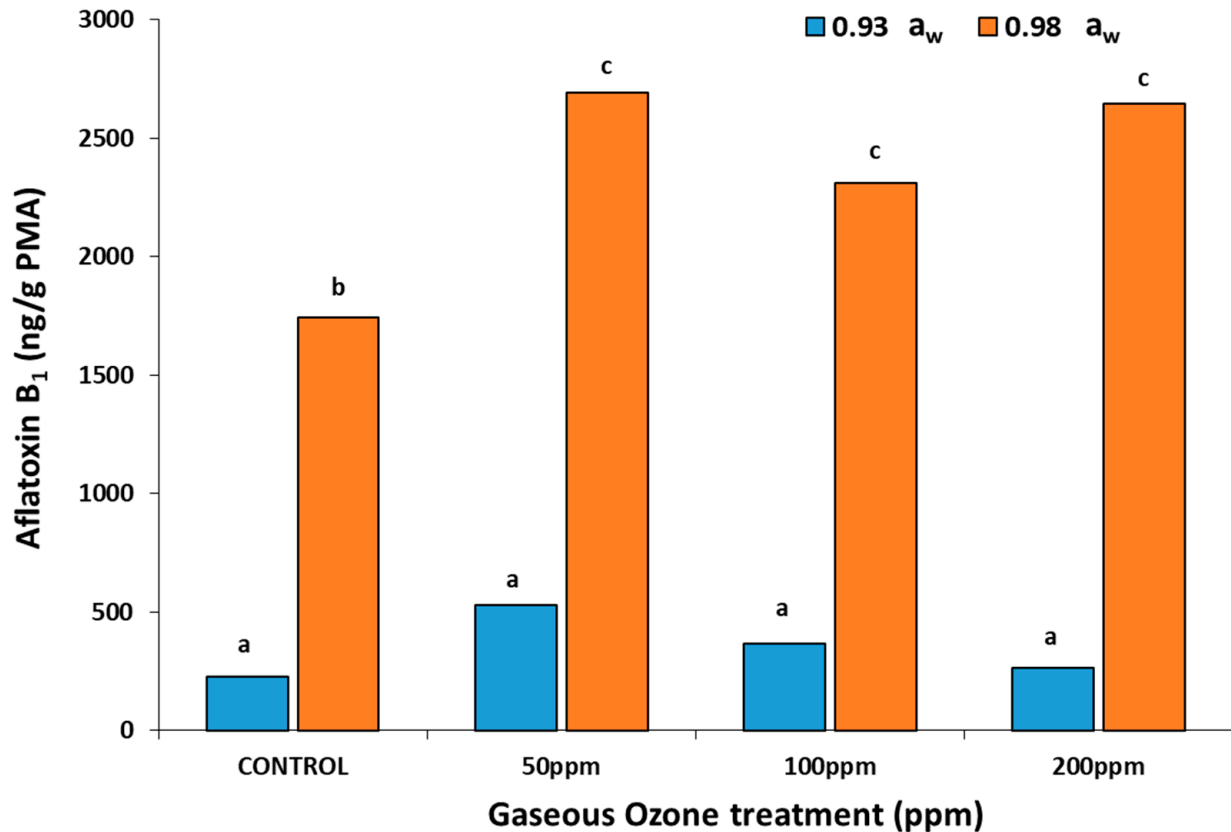


Figure 3. Effect of ozone treatment on AFB₁ production by *A. flavus* (strain AB3) at 0.93 and 0.98 a_w on a raw milled pistachio-based medium after 10 days at 30 °C. Colonies were exposed to ozone for 30 min at 6 L/min prior to incubation. Different letters indicate significant differences ($p < 0.05$).

2.4. In Situ Effects of Gaseous O₃ Treatment on Populations of *A. flavus* in Stored Raw Pistachio Nuts

The total populations of *A. flavus* slightly increased in the control samples when exposed to air (Figure 4). However, there was a significant reduction in the *A. flavus* CFUs from Log₁₀ 5.3 CFUs (control after exposure to air) to Log₁₀ 1.4 CFUs, after exposure to 50 ppm O₃, and <Log₁₀ 1.0 CFUs after 100 and 200 ppm O₃ exposure at 0.98 a_w. For pistachio nuts at 0.93 a_w, the *A. flavus* populations remained very similar regardless of the gaseous O₃ treatment. At both the a_w levels, it seemed that higher O₃ doses (100, 200 ppm) did not have any increased efficacy when compared to 50 ppm O₃ exposure.

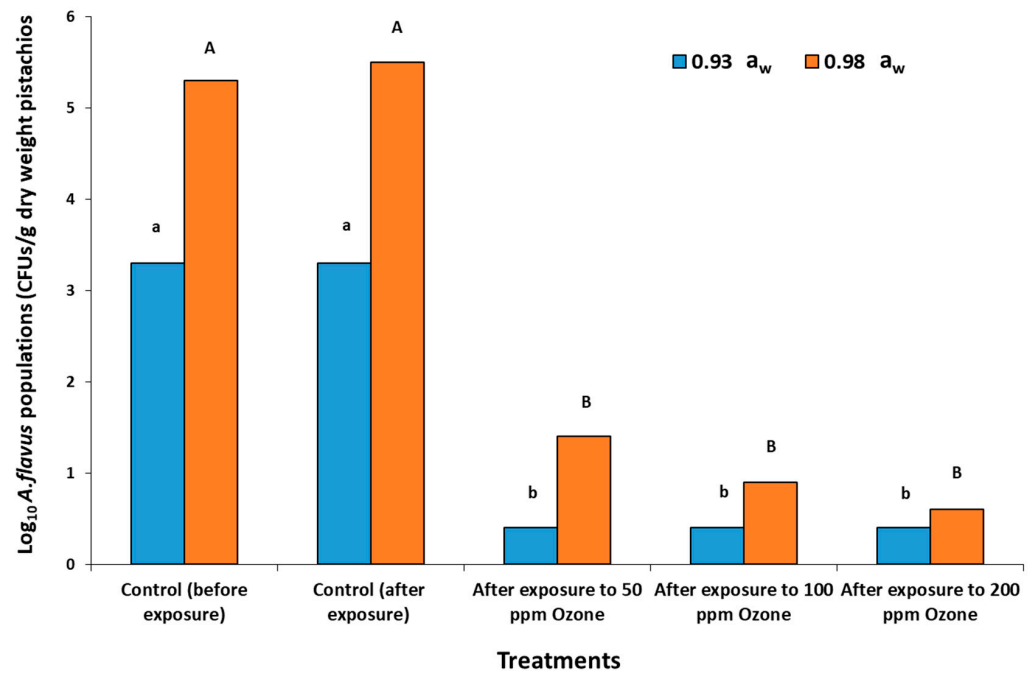


Figure 4. Populations of *A. flavus* (strain AB3, Log₁₀ CFUs) isolated from ozonized raw pistachio nut samples before and after treatment and storage for 20 days. Exposure to O₃ was for 30 min at 6 L/min prior to incubation. Different letters indicate significant differences ($p < 0.05$).

2.5. In Situ Effects of O₃ Treatment on AFB₁ Contamination of Raw Stored Pistachio Nuts

The AFB₁ contamination was found to always be higher in the treated samples compared to the controls at both a_w levels and all gaseous O₃ exposure levels, except for 50 ppm (Figure 5). At this concentration and 0.98 a_w, the AFB₁ was significantly reduced.

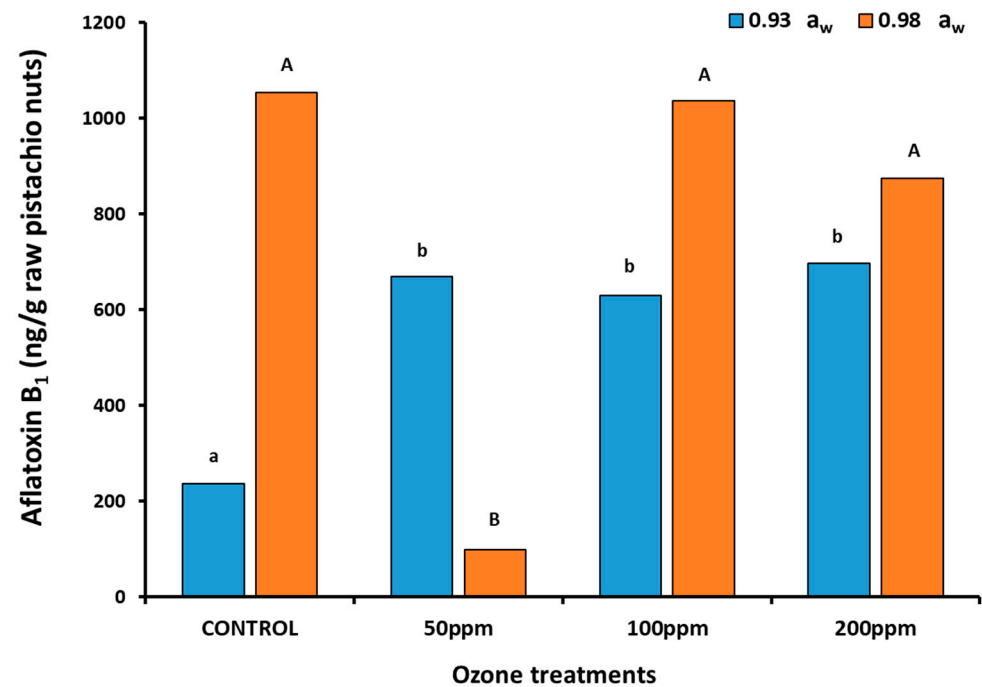


Figure 5. Effect of ozone exposure on AFB₁ contamination of raw pistachio nuts inoculated with *A. flavus* strain AB3 at 0.93 and 0.98 a_w then stored for 4 weeks after treatment at 30 °C. The pistachio nuts were exposed to gaseous ozone for 30 min at 6 L/min prior to storage. Different letters indicate significant differences between treatments ($p < 0.05$).

3. Discussion

3.1. In Vitro Effects on Germination and Mycelial Extension

The efficacy of gaseous O₃ for the control of conidial germination and mycelial growth of *A. flavus* strains (AB3, AB10) was evaluated in this study on a milled raw pistachio nut-based medium at different a_w levels. The a_w levels chosen were based on ecological data, which showed that optimum *A. flavus* growth occurs at >0.97 a_w and optimum AFB₁ at >0.94 a_w at 25–30 °C. For the prevention of toxin contamination, pistachio nuts would need to be dried to <0.88 a_w [18]. Overall, exposure of conidia to O₃ initially had lower germination percentages when compared to the controls at both the a_w levels. Treatment with 200 ppm O₃ at 0.98 a_w showed the complete inhibition of germinations after 12 h; however, spore viability appeared to recover, and the germination was increased after 24 h and reached 100% germination after 48 h. Sultan and Magan [12] found that there was an effective inhibition of conidial germination by O₃ treatment of *A. flavus* strains from peanuts with complete inhibition at 200–250 ppm O₃ (6 L/min; 30 min). However, this was on a defined yeast extract sucrose medium. Previously, Mylona et al. [14] examined the in vitro effect of O₃ treatment at 100 and 200 ppm for 30 min (6 L/min) on the spore germination of *F. verticillioides*. Although germinative capacity was inhibited after 24 h, over the following 72 h there was a recovery, and after 8–10 days at both 0.98 and 0.94 a_w, FB₁ production occurred. They also found that doubling the exposure time (60 min) did not improve the efficacy of O₃. Indeed, recent studies with species from the *Aspergillus* section *Circumdati* and *Nigri* responsible for the ochratoxin A contamination of coffee showed tolerances of up to 500 ppm O₃ [19]. It has been suggested that O₃ acts by oxidizing vital cellular components, especially unsaturated lipids in cell membranes, resulting in a leakage of cell contents and subsequent microbial lysis at high concentrations [20,21]. However, some of these studies have been performed in O₃-treated water and not with gaseous O₃ [12].

For mycelial growth, the present study showed that the mycelial extension was inhibited by O₃ exposure at 0.98 a_w. However, growth rates decreased only slightly with the increasing O₃ dose. Very few studies have examined the in vitro effect on the growth of *A. flavus*, and none were on nut-based media [12]. A previous study by Zotti et al. [22] found that O₃ treatment of 3-day-old *A. flavus* colonies for 3 h inhibited growth and spores completely. However, when the same colony reached 6 and 9 days old, the efficacy decreased. Additionally, they found that there are different sensitivity levels among species, with *A. flavus* being less sensitive than *A. niger*. However, in their study, a_w modification was not considered and the O₃ concentration used was only 1 ppm. Akbar et al. [19] found that the mycelial extension of strains of *A. carbonarius* and *A. westerdijkiae* in coffee-based medium with up to 500 ppm of O₃ had little effect on the rates of colonization, regardless of a_w level used or exposure period (30–60 min).

The general tolerance of aflatoxigenic and ochratoxigenic *Aspergillus* spp. to O₃ may in part be due to the darker pigmentation and relatively thick-walled conidia, which can provide protection against UV-light, sunlight, and toxic gases. In addition, the capacity for relatively rapid DNA repair after exposure may be quite rapid, allowing the viability of conidia to be conserved after exposure. This could be related to both pigmentation and/or repair systems that help the cells to recover viability. Indeed, Hibben and Stotzky [16] indicated that small hyaline spores are more sensitive to O₃, while large and pigmented spores, such as the conidia of *A. niger*, were more resistant [23,24]. Spores of *A. fumigatus* have been found to be particularly resistant to O₃ [18]. In contrast, spores of *Fusarium* species (e.g., *F. verticillioides*, *F. langsethiae*), which are practically hyaline and have very little pigmentation, appear to be sensitive to O₃ exposure in air initially, although some recovery of viability was found [9]. A thorough comparison amongst species belonging to the same genus is important.

Reduction of fungal growth can be obtained in high moisture conditions after treatment with up to 1000–15,000 ppm O₃ for 1 h [25]. For Brazil nuts, O₃ treatment was found to affect the growth of the mycobiota and to decrease aflatoxin contamination levels [26].

However, the exposure period was 5 h, which was effective and inhibited the growth of *A. flavus* and *A. parasiticus*, although they were still able to grow during the initial few days after O₃ exposure. Thus, O₃ levels and exposure time together with the other influencing factors, including temperature and ERH, need to be examined in more detail to optimize the potential use of this gas for control of the key life cycle phases of mycotoxigenic spoilage fungi and toxin production [21].

3.2. In Vitro and In Situ Effects of O₃ on AFB₁ Contamination

In the present study, AFB₁ was analyzed after the in vitro exposure of colonies of *A. flavus* to O₃ for 30 min and then stored for 10 days at 30 °C. There appeared to be variable effects on AFB₁ production by exposure to O₃ treatment. The increase in toxins may be due to O₃ exposure acting as an environmental stress, resulting in the biosynthesis of more toxins as a defense reaction. In addition, it may be that the O₃ interacts with the pistachio-based medium, changing the nutritional make up, especially in relation to fatty acids. Sultan and Magan [12] examined *A. flavus* exposure to O₃ (100–300 ppm) on a conducive YES medium. In this case, the use of a defined medium and exposure to O₃ resulted in a significant decrease of AFB₁ production in mycelial colonies. However, they examined toxin biosynthesis after 3 days, while the present study examined it for 10 days on a pistachio-nut based medium to simulate the natural nutritional conditions as far as was possible. This could explain the differences observed. Previously, Mason et al. [2] showed that the exposure of *A. flavus* colonies for 5 days inhibited asexual conidial sporulation. This suggested that perhaps the effect of O₃ on the whole life cycle of *A. flavus* would provide useful information on which phase might be more sensitive to such treatment [27]. The present study is the first to examine in detail the in situ effect of gaseous O₃ on the colonization and toxin production by *A. flavus* in stored pistachio nuts. Overall, while the populations of *A. flavus* significantly decreased due to O₃ exposure, there was little difference between 50–200 ppm treatment levels. Indeed, a reduction in AFB₁ was only observed in the 50 ppm O₃ × 0.98 a_w treatment. It may be that a reduction in overall populations of *A. flavus* allows more rapid subsequent colonization by the surviving inoculum of the rich nutrient source. In naturally contaminated pistachio nuts, the mycobiota is varied with a range of fungi present [28]. Thus, the niche will be occupied by a fungal community including *A. flavus*, and it would have to compete with these other fungi, some of which may survive O₃ treatment. A previous study exposed pistachio nuts and ground nuts artificially contaminated with aflatoxins to very low concentrations of O₃ (5–9 mg/L = 2–5 ppm gaseous O₃) for 140 and 420 min [10]. They found that AFB₁ and total aflatoxins were reduced by about 23 and 24%, respectively, with the highest O₃ treatment level (9 mg/L) for 420 min. The treatments were much less effective against ground pistachios [15]. However, these studies were only carried out at 70% relative humidity, and spiking with the toxins is not the same as natural occurrence in this commodity due to colonization by the mycotoxigenic species. Thus, further studies are required to better understand the relationship between commodity type, exposure concentration × time of exposure, and prevailing a_w level to determine efficacy in terms of toxin control [13].

The studies by Mylona et al. [14] certainly suggest that the natural contamination of maize grain with fumonisins can be reduced by exposure to O₃ concentrations. However, it may be more difficult to reduce mycotoxin production by specific fungi without longer term exposure to O₃. Indeed, even with 500 ppm, O₃ was found to have relatively little effect on the reduction of ochratoxin A contamination of stored green coffee contaminated with *A. westerdijkiae* and *A. carbonarius* and stored under different a_w levels at 30 °C [19]. Savi et al. [27] found that the exposure of wheat to 40–60 mg/L of O₃ for up to 180 min reduced the growth of *F. graminearum* significantly, with deoxynivalenol in the pericarp and endosperm tissue being completely inhibited. However, the moisture content of the grain was not considered in these studies, which are important relative to colonization by *Fusaria* and trichothecene production. It is, however, important to consider that food ozonation may not always be a beneficial process, especially where this gaseous treatment may alter

food sensory characteristics, color, cause lipid oxidation, and the degradation of phenolic compounds and vitamins [29,30].

4. Conclusions

This study has shown that the use of gaseous O₃ exposure of both conidia and mycelial colonies of *A. flavus* on a milled raw pistachio nut-based medium was not very effective. Indeed, via repair systems, the conidia recovered germinative capacity rapidly. Up to 200 ppm of gaseous O₃ for 30 min had little impact on mycelial extension and subsequent AFB₁ production. In situ studies with stored raw pistachio nuts inoculated with *A. flavus* conidia and exposed to O₃ reduced the isolation of the *A. flavus* populations but had little effect on AFB₁ contamination after 20 days of storage at different a_w levels. This suggests that perhaps much longer exposure times may be required and that the efficacy may also be influenced by the moisture content and the type of commodity, in this case a lipid-rich matrix. However, this would have to be balanced against potential tainting effects and eating quality, which might be impacted if high exposure levels are used for longer time periods. In the future, for more effective and safe use in food processing, the optimum gaseous O₃ concentration, contact time, and other treatment conditions need to be defined for specific foods. Pilot scale tests would probably need to be conducted for each commodity before potential commercial application, as every food application with O₃ application may be different.

In addition, in vitro and in vivo toxicological tests need to be conducted to quantify the effects of degradation products on human and animal health.

5. Materials and Methods

5.1. Apparatus for Ozone Generation and Experimental System

O₃ was generated in the laboratory using a C-Lasky series O₃ generator purchased from AirTree Ozone Technology Co. (model CL010DS), Sijhih, Taiwan. This equipment generates O₃ by corona discharge between two tubs, with no metals involved for efficiency improvement, generation stability, and less energy consumption. The generated O₃ was directed into the exposure chamber using a Teflon tube, which was properly connected to the generator. For safety reasons, the experiment was carried out in a fume cupboard to prevent O₃ from spreading into the laboratory atmosphere. Two different systems were used for O₃ exposure for in vitro and in situ assays. O₃ concentration was measured using an O₃ analyzer (Model UV-100, Eco Sensor, Santa Fe, NM, USA), which was connected to the chamber to measure the exit gas accurately. It should be noted that 1 ppm of O₃ generated is equivalent to 2.14 mg/L of O₃ in the air. This allows for comparison with some other studies. Experimental set-ups were performed as follows:

- (a) The exposure system of O₃ for in vitro germination and mycelial growth assays was a 5-L airtight glass jar. The O₃ inlet of the system was connected from the generator to the lid of the jar using a Teflon tube, which was inserted into the bottom of the jar. The outlet of the system was also in the lid of the jar and connected to the O₃ analyzer using a Teflon tube. This ensured accurate measurement of the O₃ concentrations in the glass container. The flow rate of the generated O₃ used was 6 L/min for 30 min.
- (b) Exposure system of O₃ for the in situ study.

The exposure chamber for in situ experiments was a 100-mL volume glass tube. The tube was capable of containing about 45–50 g of pistachio nuts. These were placed inside the column, and the O₃ was forced upwards via an inlet at the bottom of the tube coming from the generator. The outlet at the top was connected to the O₃ analyzer and vented in the fume hood. This allowed accurate exposure of the pistachio nuts to the treatment O₃ concentrations for the necessary residence time. The flow rate of generated O₃ was 6 L/min for 30 min.

5.2. Fungal Strains, Media, Spore Suspension, and Water Activity

One strain of *A. flavus* (AB3), representative of 3–4 others isolated from pistachio nuts, was used in these studies [13,31]. This was chosen as it was representative of those studied previously [13,31]. Pistachio-based media were used for spore germination and mycelial growth studies. A 3%-milled raw pistachio nut agar (PMA) was used with 2% technical agar (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, Hampshire, UK) [13].

For spore suspension, fresh cultures of the AB3 strain were prepared on PMA and incubated at 25 °C for 5–7 days. AB3 culture surfaces were gently scraped and transferred into sterile Universal vials containing sterile water + 0.1% Tween 80 solution (Tween 80 (ACROS organics). The concentration of the spore suspension was determined using a hemocytometer (Olympus BX40 microscope, Microoptical Co., Sauerlach, Germany; slide Marienfeld superior, Germany; microscope glass cover slips, No 3, 18 mm × 18 mm, Chance proper Ltd., Malvern, Worcestershire, UK) and adjusted by dilution to 10⁷ spores/mL. Target a_w values (0.98 and 0.93) for PNA were obtained by using glycerol/water solutions, instead of water, to modify the a_w with this non-ionic solution. For these two treatments, the equivalent of 122.5 and 355 g of glycerol per L of water was used. This was mixed, and the mixture was used similar to water.

5.3. Effect of O₃ on Conidial Spore Germination of *A. flavus*

Four different treatments were examined, including three concentrations of O₃: 0, 50, 100, 200 ppm of O₃ at the two different a_w levels detailed previously and incubated at 30 °C. Samples exposed to the air were used as controls for each a_w . The experiment was carried out in triplicate and repeated once. An amount of 100 µL of 10⁶ spore suspension was spread onto PNA media treatments and replicates and allowed to dry. Lids were taken off the plates and the media were placed inside the airtight glass jar for O₃ exposure for 30 min at a flow rate of 6 L/min, as described previously. The Petri plates were separated by 2–3 cm to ensure exposure of each plate. After exposure, plates were placed into plastic boxes, which were maintained at the same a_w levels with glycerol/water solutions (500 mL × 2) and stored at 30 °C. Three agar plugs were taken every 12 h from each plate using a surface-sterilized cork-borer (1 cm) and placed on a glass microscope slide (Fisherbrand, Leicestershire, UK). The agar plugs were then stained with Lactophenol Cotton Blue (ProLab Diagnostics, Birkenhead, UK) and covered with a glass coverslip. Each plug was then examined under the microscope, and germination was recorded. Spores were considered to have germinated when the length of the germ tube was longer than the diameter of the spore. A total of 3 × 50 single spores per replicate were examined (450 per treatment). The overall mean number of germinated spores (out of 50) was calculated for the different O₃ treatments.

5.4. In Vitro Effects of Gaseous O₃ on Mycelial Growth and AFB₁ Contamination

PNA media were inoculated centrally with 10 µL of spore suspension made from AB3 strain and incubated at 30 °C in replicates and allowed to grow for 2 and 5–6 days in the 0.98 and 0.93 a_w treatments, respectively. Measurements of colonies were recorded, and plates were exposed to O₃ with no lids for 30 min using the system described previously. The O₃ concentrations were 50, 100, and 200, with air as a control. A_w of media and ERH during incubation after O₃ exposure was adjusted to 0.93 and 0.98 a_w . After exposure, Petri plates were covered with the lids and incubated at 30 °C. Colony diameters were recorded on a daily basis for each treatment and compared with the control. Agar plugs were taken after day ten from each replicate and stored at −20 °C for AFB₁ analysis.

5.5. In Situ Effect of Gaseous O₃ on Fungal Population and AFB₁ Production on Irradiated Pistachio Nuts

Irradiated raw pistachio nuts (12–15 KGys; Synergy Health Sterilisation UK Ltd., Swindon, Wiltshire, UK) were weighed and placed in sterilized bottles (eight bottles) for each treatment (40 g per replicate). The absence of any fungal contaminants was checked

by direct-plating individual pistachio nuts on Malt Extract Agar medium (MEA; Thermo Fisher Scientific Oxoid Ltd., Basingstoke, Hampshire, UK) and incubated for 7 days at 25 °C. This showed no contamination. The raw pistachio nuts were rewetted using a moisture adsorption curve [25] and mixed well and left overnight at 4 °C to equilibrate to the target a_w levels of 0.93 and 0.98. A conidial suspension of 10^6 spores was prepared. After equilibration, 1 mL of the spore suspension was added to the pistachio nuts and mixed well. Small sub-samples were taken (1 g) and placed in 10 mL of sterile water containing tween 80 in a 25 mL Universal bottle for serial dilution to assess the populations of *A. flavus* present. Three separate replicates of each treatment (40 g each) were exposed to O₃ (50, 100, and 200 ppm, or air) for 30 min at a flow rate of 6 L/min.

Immediately after exposure, sub-samples were taken for serial dilution of the populations. The rest of the pistachio nuts were placed in solid culture vessels with microporous lids (Magenta, Sigma Ltd., Coventry, UK). These were previously autoclaved at 121 °C for 15 min with aluminum foil covers. The glass chambers containing the treatments/replicates were then placed in plastic chambers with glycerol/water solutions to maintain the target ERH (93 and 98% ERH) and stored for 20 days at 30 °C. Samples were taken for *A. flavus* fungal populations after this storage period. The remaining pistachio nuts were stored at −20 °C for later AFB₁ analysis.

For serial dilution, samples were soaked for 20 min and then vigorously shaken using a vortex mixer. From each treatment/replicate serial dilutions were made. For each concentration, three replicates were made, and 100 µL was spread on MEA media using a sterile spreader and incubated at 30 °C for 7 days before colonies were counted for *A. flavus* populations.

5.6. Aflatoxin B₁ Quantification

Preparation of aflatoxin standards: A 200-µL stock solution of aflatoxins (B₁, B₂, G₁, G₂) standard in methanol containing 250 ng AFB₁ was prepared and pipetted into 2-mL Eppendorf tubes for overnight evaporation until dryness in a fume hood similar to the samples.

5.6.1. In Vitro Aflatoxin B₁ Analyses

Colony Extraction: Initially, agar plugs were cut out across the diameter of colonies using a surface-sterilized 4-mm diameter cork-borer (approx. 4–6). The agar plugs were placed in pre-weighed 2-mL Eppendorf tubes and weighed again. Five-hundred µL of HPLC-grade chloroform was added to the tubes and shaken for 30 min using a KS 501 digital orbital shaker (IKA (R) Werke GmbH & Co. KG, Esslingen, Germany). The chloroform extract was transferred to a new Eppendorf tube and dried gently under air for derivatization.

Derivatization of aflatoxin B₁ extract: Derivatization of the AFB₁ extract was performed according to the AAOC method [32]. First, 200 µL of hexane was added to the tube, followed by 50 µL of trifluoroacetic acid. The mixture was vortexed for 30 s and left for 5 min. A mixture of water:acetonitrile (9:1) was then added to the tube, vortexed for 30 s, and left for 10 min to allow for separation of the layers. Then, the aqueous layer was filtered using a syringe nylon filter (13 mm × 0.22 µm; Jaytee Biosciences Ltd., Herne Bay, UK) into amber-salinized 2-mL HPLC vials (Agilent, Santa Clara, CA, USA) before HPLC analysis. All analytical reagents used were HPLC-grade.

Quantification of aflatoxin B₁ with High Performance Liquid Chromatography HPLC: A reverse-phase HPLC with fluorescence detection was used to confirm the identity and quantify AFB₁. An Agilent 1200 series HPLC system was used for the analysis. It consisted of an in-line degasser, auto sampler, binary pump, and a fluorescence detector (excitation and emission wavelengths of 360 and 440 nm, respectively). Separation was achieved using a C18 column (Phenomenex Gemini; 150 × 4.6, 3 µm particle size; Phenomenex, Torrance, CA, USA) with a Phenomenex Gemini C18 3 mm, 3-µm guard cartridge. Isocratic elution with methanol:water:acetonitrile (30:60:10, *v/v/v*) as the mobile phase was performed at a flow

rate of 1.0 mL/min. The injection volume was 20 µL. A set of standards was injected (1 to 5 ng AFB₁, AFB₂, AFG₁, and AFG₂ per injection), and standard curves were generated by plotting the area underneath the peaks against the amounts of AFB₁ standard injected. The run time for the each HPLC analysis was 12 min. Supplementary Figure S1 shows an example of a standard and a sample for aflatoxin B₁ quantification.

5.6.2. Quantification of Aflatoxin B₁ in Pistachio Nuts

The pistachio nut samples were all dried in a drying oven at 50 °C in the dark. They were subsequently ground (Waring blender, Merck Ltd., Feltham, UK) and weighed (25 g). The background aflatoxin B₁ level in the nuts used in the experiments was 0.015 ng/g. This was taken into account as a correction factor in the final quantification of the results. Acetonitrile/water 60/40 (100 mL) was used as an extraction solvent. The mixture was blended for 3 min, and the extract was filtered into a smaller sample container. PBS buffer (pH 7.4, Thermo Fisher Scientific) was used for sample dilution, then the diluted extract was passed through an Immunoaffinity Column (IAC; AflaStar™; Romer Labs, Tulln an der Donau, Austria) with a flow rate between 1–3 mL/min. The column was rinsed with 2 × 10 mL sterile distilled water. HPLC-grade methanol (1.5–3 mL) was then applied to the column, and the eluent was collected in a new amber glass vial and left to dry overnight at room temperature before the derivatization step, as detailed previously.

5.7. Statistical Analysis

Three replicates per treatment were used in all studies and repeated once. Means were obtained by taking the average of three measurements for each experiment with the standard error of the means (±SE; standard error) obtained. Analysis of variance (ANOVA) was applied to analyze the variation of means with a 95% confidence interval. Normal distribution of data was checked by the normality test Kolmogorov-Smirnov, using Minitab statistical software. Fisher's Least Significant Difference (LSD) was used to identify differences between the means, with $p < 0.05$ as a significant difference, using the same statistical software.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/toxins14060416/s1>, Figure S1: Examples of HPLC analyses of (a) a standard and (b) a sample of pistachio nut-based agar for aflatoxin B₁ quantification.

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