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Decontamination of maize kernels and degradation of mycotoxins by means of cold plasmas

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ABSTRACT

In most of the maize-cultivated areas, contamination by mycotoxigenic species poses phyto pathological concerns and toxicological risks for humans and animals, leading to significant economic losses and compromised food safety for both unprocessed maize and its derived products. Various strategies are currently employed worldwide to reduce fungal contamination responsible for mycotoxin production. In this research we have tested the efficacy of cold plasma processes in controlling the growth of *Aspergillus flavus* and *Fusarium proliferatum*, as well as the degradation of their mycotoxins. Two modalities of remote plasma application and rinsing with plasma-treated tap water were compared. The treatments used in this research resulted in a reduction of conidial germination and subsequent growth on maize by 50%–90% for both species. Air-fed plasmas proved to be the most effective against *A. flavus* and *F. proliferatum*. Furthermore, reductions in mycotoxin levels were observed after an O_2-fed plasma treatment on naturally contaminated maize kernels, with variable efficacy against fumonisin B_1 (FB_1) and aflatoxin B_1 (AFB_1). Moreover, plasma degradation products of AFB_1 were investigated and identified. This study demonstrates the potential of cold plasma-based treatments as a post-harvest decontamination approach to reduce fungal infections and mycotoxin contamination during storage and packaging.

1. Introduction

Maize, among the most cultivated crops worldwide, plays a key and increasing role in global agri-food systems including direct food consumption and indirect feed pathways for animal-sourced foods (Erenstein et al., 2022). In all maize-cultivated areas, specific pedoclimatic conditions can lead to both abiotic and biotic stresses, resulting in significant economic losses and threatened food safety for both unprocessed maize and its derived products. Among biotic stresses, mycotoxigenic fungi are of particular concern, as they can colonize maize during the entire crop cycle and the post-harvest storage.

The most frequently occurring mycotoxigenic fungal species belong to the genera *Fusarium* and *Aspergillus*. *Fusarium proliferatum*, along with *F. verticillioides*, is associated to some of the most devastating fungal diseases of maize, including *Fusarium* ear and stalk rots, and *Fusarium* stem and root rots. *Aspergillus flavus* is also commonly detected on maize kernels in maturity and during storage. Although primarily found in tropical regions, this species has increasingly been reported in various areas of Europe due to recent climatic changes (Camardo Leggieri et al., 2021). Contamination by *Fusarium* and *Aspergillus* species poses phyto-pathological issues and toxicological risks for humans and animals. Many of these species produce a wide range of mycotoxins, harmful secondary metabolites, that accumulate in maize kernels and often remain stable even after physical and chemical treatments, including high temperature used in food processing. The mycotoxins most frequently found worldwide on maize are fumonisins (FBs), particularly FB_1, FB_2, and FB_3 types, produced mainly by *F. verticillioides* and *F. proliferatum*, and aflatoxins (AFs), produced by *A. flavus* and, to a lesser extent, by other *Aspergillus* species (Kamle et al., 2019). Moreover, *F. proliferatum* is ubiquitous, capable of colonizing a wide range of important agricultural crops besides maize and other cereals, including vegetables and fruit trees (Proctor, Desjardins, & Moretti, 2009). This

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species produces not only FBs, but also other mycotoxins such as moniliformin (Marasas et al., 1986), beauvericin (Moretti et al., 1994), fusaproliferin (Ritieni et al., 1995), and fusarins (Miller et al., 1995), thus increasing the risk of multiple mycotoxin occurrence in foodstuff. Among FBs, FB_1 is classified by the International Agency for Research on Cancer (IARC) as possible carcinogen for humans (Group 2B; IARC, 2002). Additionally, FBs can induce several significant health effects in livestock and other animals (Damiani et al., 2019). In contrast AFs, known for their acute toxicity, carcinogenicity, mutagenicity, teratogenicity, and immunosuppressive properties, are classified as Group 1 carcinogens for humans (Ostry et al., 2017). To address the toxic effects of FBs and AFs on humans and animals, European authorities have established maximum levels for these mycotoxins in food and feed to ensure safety (EC, 2006; 2007; EFSA, 2018).

In line with Integrated Pest Management strategies, various agronomic, genetic and biological techniques, as well as agricultural practices, are now available to prevent or limit fungal diseases and associated mycotoxin accumulation. Different chemical and physical strategies are used globally to combat crop contamination and reduce fungal species responsible for mycotoxin production. However, currently used decontamination procedures often have drawbacks, such as loss of nutritional properties and high environmental impact, which limit their application in the food industry (Karlovsky et al., 2016). Moreover, since the biological and chemical detoxification of mycotoxins is not permitted for human food within the European Union, physical methods are the only approved approaches in food industry (Liu et al., 2024). This opens the way to the research of newer physical detoxification methods.

Cold plasmas are partially ionized gases that include electrons, negative and positive ions, highly reactive molecules and free radicals generated by the fragmentation of a feed gas. When maintained in non-equilibrium conditions, cold plasmas can operate at room temperature and are suitable for a wide range of technological applications (Weltmann et al., 2019). When ignited at atmospheric pressure, these glow discharges are referred to as cold atmospheric plasma (CAP). Due to their versatility, low environmental impact, rapid treatment speed, and ease of integration in industrial processes, CAP technologies offer promising alternatives to conventional decontamination methods (Katsigiannis et al., 2021, 2022; Misra et al., 2019).

When a plasma is fed with O_2, N_2 or mixtures of them, including air, reactive oxygen and nitrogen species (RONS) are produced. The most commonly found reactive species in atmospheric air CAPs are O, OH, O_2^*, O_3, O_2^-, HO_2, H_2O_2, N, N_2^-, NO, NO_2, NO_3, and N_2O. When CAPs interact with moisture, H_2O_2, NO_2^-, NO_3^- and $ONOO^-$ species are generated; these are considered long-lived reactive species with extended stability in aqueous phases (Gorbanev et al., 2018; Veronico et al., 2022). It is widely believed that plasma-assisted food decontamination processes are initiated by RONS, creating a highly reactive environment that induces various changes in exposed targets. The decontamination efficiency is influenced by multiple factors, including feed gas, process parameters and plasma exposure mode (e.g., direct vs. indirect) (Hernández-Torres et al., 2022).

Most literature focuses on the direct exposure of contaminated samples to CAPs (Sakudo et al., 2019; Ganesan et al., 2020), where the target is completely immersed in the ionized gas and becomes part of the electric circuit, influencing the electrical properties of the discharge. This allows the target to benefit from the synergistic effects of direct UV radiation and short- and long-lived reactive species against fungi and/or mycotoxins, resulting in highly efficient processes (Liu et al., 2024; Ma et al., 2015; Mravlje et al., 2021; Ten Bosch et al., 2017). The primary mechanisms of microbial destruction involve the breakdown of cell walls through reactions of RONS with peptiglycan layers, deterioration of cell membranes due to hyperoxidation of unsaturated fatty acids and proteins primarily attributed to OH radicals, and DNA oxidation.

In direct exposure modes, UV light and temperature are thought to have minimal effects on decontamination (Mravlie et al., 2021; Wannicke et al., 2021). Indeed, increased UV light and heat can compromise product quality and organoleptic properties, potentially altering the genetics of kernels (Peťková et al., 2021; Katsigiannis et al., 2022). Furthermore, kernels germination time may be affected, posing ecological and safety risks to the final product (Priatama et al., 2022). Thus, it is crucial to develop milder approaches that maximize the beneficial decontaminating effects of plasma and minimize possible damage.

The referenced studies highlight growing interest in CAPs for food decontamination. Although several papers have reported the effects of direct CAPs on fungal species responsible for food spoilage and their metabolites, including mycotoxins (Hoppanová & Kryštofová, 2022; Veerana et al., 2022; Ranjan et al., 2023), most of these studies deal with *in vitro* experiments. However, recent advances on the application of CAPs vs mycotoxin degradation are available, providing discussion on possible working mechanisms and their effect on food quality, and on the toxicity of degradation products (Liu et al., 2024; Wang et al., 2023; Xiang et al., 2021).

The present paper shows that, besides the effects of UV radiation and electrical field, the RONS produced in N_2 and/or O_2 fed CAPs play a pivotal role in mycotoxin degradation, as stated also by Liu et al. (2024). To evaluate the effectiveness of RONS in kernel decontamination, we decided to test plasma processes fed with either pure O_2 or synthetic dry air. To enhance versatility and efficiency of these processes and minimize the risk of altering food quality, we opted to test the effect of a remote configuration, switching on the plasma far from the target, as well as to evaluate the decontamination effects induced by plasma-treated water (Cherif et al., 2023; McQuaid et al., 2023).

The aim of this study is to explore alternative decontamination approaches for maize kernels and methods for mycotoxins degradation aided by soft plasma technologies. Additional aims are the investigation of the pattern of the degradation products of mycotoxins derived from plasma treatments, and the application of plasma technologies on naturally contaminated maize kernels.

2. Materials and methods

2.1. Chemicals

Analytical-grade methanol (MeOH) was purchased from Mallinckrodt Baker (Milan, Italy). UPLC grade Acetonitrile was obtained from Merck (Merck KGaA, Germany). Ultrapure water was produced in house with a Millipore Milli-Q system (Millipore, Bedford, MA, USA). The (3*S*,7*R*)-11-methoxy-6,8,19-trioxapentacyclo[10.7.0.0^{2,9}.0^{3,7}.0^{13,17}] nonadeca-1,4,9,11,13(17)-pentaene-16,18-dione ($C_{17}H_{12}O_6$; AFB1 purity >99 %), the (2*R*)-2-[2-[(5*S*,6*S*,7*R*,9*R*,11*S*,16*S*,18*R*,19*R*)-19-amino-6-[(3*S*)-3,4-dicarboxybutanoyl]oxy-11,16,18-trihydroxy-5,9-dimethylicosan-7-yl]oxy-2-oxoethyl]butanedioic acid ($C_{34}H_{59}NO_{15}$, FB_1, purity >99 %) and NaCl were purchased from Sigma (Milan, Italy). Filter paper grade 4 (20–25 μm particle retention) and glass microfiber filters (GF/A) were purchased from Whatman International Ltd. 132 (Maidstone, UK). AOF MS-PREP® immunoaffinity columns were obtained by Romer Lab (Romer Labs Inc., Tulln, Austria). The Phosphate Buffered Solution (PBS) at pH 7.4 was prepared by dissolving commercial PBS tablets (Sigma–Aldrich, Milan, Italy) in distilled water. Regenerated cellulose membrane filters (RC 0.2 μm) were obtained from Phenomenex (Bologna, Italy).

2.2. Plasma processing

Three different treatment approaches were utilized on conidial suspensions of *A. flavus* and *F. proliferatum* plated on potato dextrose agar (PDA) medium or directly applied to maize seeds, namely: 1) remote plasma processing, where samples were positioned a few millimeters away from the plasma phase (Close Plasma treatment, CP); 2) introducing a stream of plasma-treated gas (Plasma Treated Gas, PTG) into a

container with the contaminated samples; 3) processing the samples with plasma-treated tap water (Plasma Treated Water, PTW). For all tests three samples per approach were treated once, on the same day, marking the time zero of the storage period.

CAPs were generated with a modified Dielectric Barrier Discharge (DBD) PetriPlas + plasma source, described in detail elsewhere (Ricciardi et al., 2022; Veronico et al., 2021, 2022). The source consists of a Plexiglas flow unit equipped with gas connections and a discharge unit. A dry diaphragm pump (Pfeiffer Vacuum, Aβlar, GER) is connected directly to the flow unit for the CP approach and during the production of PTW, or through the sample holder containing the Petri dish with maize kernels in the case of PTG (Fig. 1b). The pump is used to evacuate exhaust gases and maintain the constant pressure of about 760 Torr, as measured with a MKS baratron.

An electric field of 6 kHz frequency and 13.8 kV peak-to-peak voltage was generated using a power supply connected to a programmable 10 MHz DDS function generator (TG1010A, Aim-TTi, Huntingdon, UK) to ignite a volume discharge of the size of the high voltage (HV) electrode (3 cm dia). The DBD discharges were ignited for 5 min, pulsed with a 50% duty cycle (D.C.), 50 ms of plasma on (t_{on}) over a period ($t = t_{on} + t_{off}$) of 100 ms. In the CP and PTW modes the discharge unit was adapted for treating maize kernels and tap water, respectively, contained in a Petri dish (6 cm dia) fastened under the DBD source, in a slot of the same diameter of the flow unit (Fig. 1a–b). In both cases, the distance between the plasma and the surface of the sample to be treated was set at 2 mm.

For the PTG treatments, a glass Petri dish was positioned beneath the DBD, allowing the exhaust gas, along with its long-lived species, to flow through an external holder containing a Petri dish filled with agar or maize kernels (Fig. 1c). All processes for the three approaches were carried out in a closed system, not exposed to the surrounding atmosphere; the reaction chamber was purged with the designed gas feed for 1 or 2 min before plasma ignition, depending on the approach used.

PTG and CP treatments of maize kernels were facilitated by diffusing RONS from the CAP to the samples through the grid, grounded, of the DBD (Fig. 1b). Pure O_2 (air liquid, 99.999%) and synthetic air (air liquid, 99.999%) were used for the treatments. Gas flow rates were measured with MKS mass flow controllers.

Table 1 summarizes the experimental conditions utilized in the plasma treatments, where each condition is denoted with a name that combines the treatment approach (CP, PTG or PTW) with the gas feed and treatment duration (es. CP-O_2 3 min).

2.3. Determination of the effects of plasma and reactive species on *Aspergillus flavus* and *Fusarium proliferatum*

2.3.1. Artificial contamination of potato dextrose agar (PDA) medium and maize kernels and evaluation of conidial germination

Two aflatoxigenic *Aspergillus flavus* strains, ITEM 8095 and ITEM 8111, along with a single *Fusarium proliferatum* strain, ITEM 12072, were used to assess the effects of different plasma treatments on conidial germination. These strains, isolated from Italian maize, were selected within the ITEM microbial collection of the Institute of Sciences of Food Production (CNR-ISPA), for their ability to produce high levels of aflatoxins and fumonisins under both field conditions and *in vitro*.

For each strain, 100 μL aliquots of conidial suspension, along with three decimal dilutions were spread on PDA plates and subsequently exposed to CAPs in triplicate. Three untreated inoculated plates served as control. The conidial germination rates of control and plasma treated samples were analysed after 48 h of incubation at 25 °C.

To evaluate the effects of CAP treatments on maize kernel surface-contaminated with conidia, the kernels were first sterilized in autoclave. They were then inoculated after 1 min immersion in 100 μL conidial suspension (10^7 conidia/mL) and allowed to dry in microbiological hood at room temperature. For the CP and PTG treatments, maize samples artificially contaminated with fungal spores were placed in Petri dishes (40 kernels per dish). After the treatment, each sample was

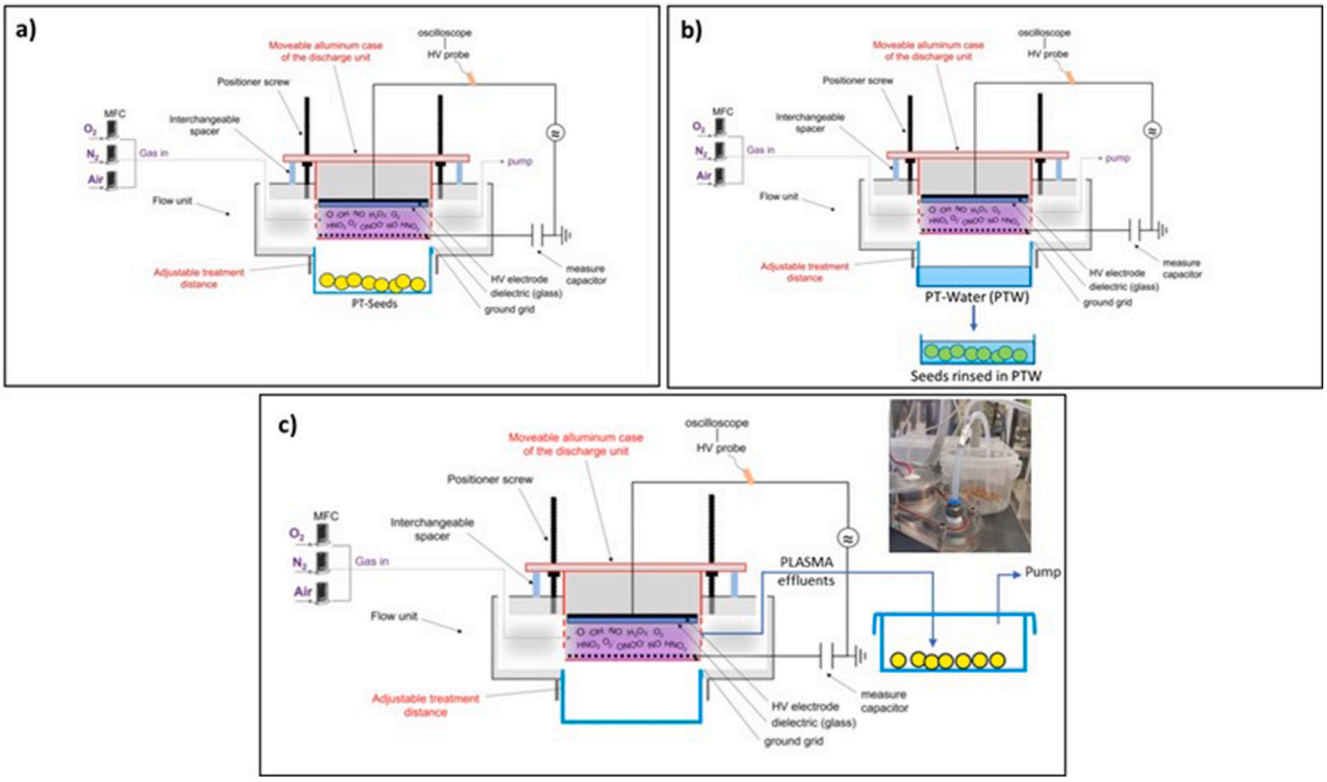


Fig. 1. Schematic representations of cold atmospheric plasma treatment of maize kernels with a volume Dielectric Barrier Discharge (DBD) used in three different remote approaches: (a) Close Plasma treatment (CP), with the sample underneath the plasma source; (b) Plasma Treated tap Water (PTW), with the samples rinsed in tap water previously exposed to CAP; c) Plasma Treated Gas (PTG), with the samples exposed downstream to gas effluents from CAP.

Table 1
Experimental conditions used for plasma treatments of fungal conidia (agar medium-PDA, maize kernels) and AFB_1 and FB_1 mycotoxins (paper, maize kernels).

Treatment	Typology of sample	Duty Cycle (%)	Peak to peak voltage (kV)	Frequency (kHz)	Gas feed and flow (SLM)	Purging time (min)	Treatment time (min)
CP	Maize kernels (n°40 or 5 g[a]), PDA, paper	50	13	6	O_2 or Air (0.5 SLM)	1	3, 20
PTG	Maize kernels (n°40 or 5 g[a]), PDA, paper	50	13	6	O_2 or Air (1 SLM)	2	3, 20
PTW	Maize kernels (n°40), PDA sprayed with 2 mL of tap water	50	13	6	O_2 or Air (0.5 SLM)	1	5

[a] Amount of kernels used to quantify mycotoxin's degradation; SLM: standard liter per minute.

rinsed with 10 mL sterile distilled water to obtain conidial suspensions, which were then properly diluted and spread (100 μL) onto PDA plates.

For testing the activity of PTW, tap water instead of sterile distilled water was plasma treated to prepare conidial suspensions. A conidial suspension in sterile distilled water was used as control.

2.3.2. Evaluation of the effects of plasma treatments on conidial germination

After 48 h of incubation at 25 °C the germinated conidia capable of forming fungal colonies on the PDA plates were counted. The effect of plasma treatments on germination was assessed by comparing the number of colonies in each treated group with the number of colonies in the controls (untreated theses). Each reported value represents the mean of three replicates. The inhibition of germination, expressed as a percentage, was calculated using **Eq. (1)**:

$$\text{Inhibition } (\%) = [(C - T) / C]\text{X } 100 \qquad \text{Eq.1}$$

where C is the number of colonies grown in the control sample and T represents the number of colonies grown in the treated sample.

2.4. Evaluation of the effect of plasma on AFB_1 and FB_1 mycotoxins

2.4.1. Preparation of mycotoxins standard solutions

Stock 1 mg/mL solutions of AFB_1 and FB_1 were prepared by dissolving solid commercial toxins in acetonitrile and a 50:50 (v/v) acetonitrile/water solution, respectively. The AFB_1 solution was then diluted to 1 μg/mL in acetonitrile, while the FB_1 solution was diluted to 100 μg/mL in the acetonitrile/water solution. These solutions were utilized for spiking experiments on maize kernels and paper filter to evaluate the degradation efficiency of the various treatments. Quantification of mycotoxins was performed using a matrix-matched calibration curve for the detection on maize kernels (20–100 μg/kg for AFB_1 and 100–1000 μg/kg for FB_1) and a standard calibration curve for the detection on paper (0.02–0.10 μg/mL for AFB_1 and 0.1–1.0 μg/mL for FB_1).

2.4.2. Contamination of filter paper and maize kernels with AFB_1 and FB_1 standard solutions

The effect of plasma exposure on AFB_1 and FB_1 was evaluated independently using distinct samples on both filter paper and maize kernels. For each mycotoxin, a set of 18 samples was prepared and analysed: 3 contaminated samples for each plasma treatment (CP-O_2, CP-Air, PTG-O_2, PTG-Air); 3 contaminated untreated samples (positive control); 3 uncontaminated plasma treated samples of maize kernels (negative control).

Filter paper and maize kernels (5 g) were artificially contaminated with 0.25 mL of AFB_1 solution (1 μg/mL) and 0.05 mL of FB_1 solution (100 μg/mL). This resulted in final concentrations of 0.05 μg/mL on paper and 50 μg/kg on maize kernels for AFB_1, as well as 1 μg/mL on paper and 1000 μg/kg on maize kernels for FB_1. To detect potential AFB_1 degradation products, which are likely to be present in much smaller amounts than the original compounds, and considering the instrumental sensitivity, it was necessary to select a level of AFB_1 contamination in maize kernels one order of magnitude above the maximum allowed limit in food and feed. The samples were allowed to rest at room temperature for approximately 2 h to facilitate the evaporation of the solvent.

2.4.3. Mycotoxin analysis by LC-HRMS

The quantification of mycotoxins in filter samples was performed after extraction with 3 mL of methanol/water (70:30, v/v). The samples were filtered through a 0.22-μm RC syringe filter before injection into the LC-HRMS apparatus.

For quantifying the mycotoxins in kernel samples, AOF MS-PREP® immunoaffinity column cleanup was run according to the manufacturer instructions. Briefly, 5 g of kernel samples and 1 g of NaCl were extracted with 20 mL of methanol/water (60:40, v/v), blended at high speed for 2 min. After centrifugation (4000 rpm, 10 min), 10 mL of the filtrate were diluted with 15 mL of PBS. Following filtration through glass microfiber filter paper, 5 mL of the filtrate (equivalent to 0.5 g of sample) were injected in the column, previously rinsed with 20 mL of water. The toxins were eluted with 1 mL of methanol followed by 1 mL of water. The final sample (10 μL) was then injected in the UHPLC-HRMS/MS equipment.

For analysing degradation products, additional samples of treated and control maize kernels were prepared and extracted with 20 mL of methanol/ultrapure water (80:20, v/v) in orbital shaker for 1 h. The extracts were then diluted tenfold with water, centrifuged (15000 rpm, 4 °C, 20 min) and injected in the UHPLC/HRMS equipment.

Liquid chromatography–HRMS analysis was performed with a Q-ExactiveTM Plus mass spectrometer, equipped with a heated electrospray ion source (HESI II) coupled to an Ultimate 3000 UHPLC system (all from Thermo Fisher Scientific, San Jose, CA, United States). A Gemini UHPLC C18 column (150 × 2 mm, 5-μm particles, Phenomenex, Torrance, CA, United States) was used, preceded by a Gemini C18 guard column (4 × 2 mm). The column oven was set at 40 °C, the injection volume was 20 μL. The flow rate of the mobile phase was set at 200 μL/min, with eluent A water and eluent B methanol, both containing 0.5% acetic acid and 1 mM ammonium acetate. For creating the gradient, the proportion of eluent B was kept constant at 10% for 5 min and then linearly increased to 80% in 36 min. Finally, it was raised to 100% and kept constant for 5 min. The column was re-equilibrated with 10% eluent B for 9 min. The HESI II ion source was operated both in positive and in negative ion modes, with parameter settings reported in Ciasca et al. (2020).

AFB_1 and FB_1 degradation ratio (Mycotoxin degradation %) and degradation standard deviation (Degradation SD) were evaluated using respectively Eq. (2) and Eq. (3):

$$\text{Mycotoxin degradation } \% = 100*[\text{AreaC- AreaT}]/\text{ AreaC} \qquad \text{Eq.2}$$

$$Degradation\ SD = Area\ T \Big/ Area\ C * \sqrt{(SDT/AreaT)^2 + (SDC/AreaC)^2} \qquad \text{Eq.3}$$

AreaC is the peak area in the AFB1 (or FB1) control sample; AreaT is the peak area in the AFB1 (or FB1) treated sample; SDT is the standard deviation for AFB1 (or FB1) treated samples; SDC is the standard deviation for AFB1 (or FB1) control samples.

2.5. Statistical analyses

The standard deviation values for all theses were calculated, based on three replicates. The one-way analysis of variance (ANOVA) was used to determine whether there are any statistically significant differences between the treatments. The means were then compared using the Tukey's multiple comparisons method as post-hoc test. If the p-value for the 95% confidence interval was below 0.05, differences were considered significant. Statistical analyses were performed using GraphPad version 10.0.0 software.

3. Results

3.1. Activity of the plasma treatments on Aspergillus flavus

The quantification of the fungal colonies developed on PDA after each treatment compared to the untreated controls was used to calculate the inhibition of germination induced by the plasma treatments. The results shown in Figs. 2 and 3 are derived from at least six different experiments in all treatment conditions. The data for *A. flavus* result from experiments run on two different strains showing a high level of reproducibility.

Fig. 2 shows the results of conidia spread on PDA before the treatments. All treatments were found to effectively reduce conidial germination, with inhibition values ranging from 35% (PTW) to 100% (PTG). Specifically, PTW exhibited a notable but lower and significant ($P < 0.0001$) efficacy compared to the other treatments, with about 44% inhibition. In contrast, both CP and PTG treatments resulted in 100% inhibition of germination for *A. flavus*, regardless of gas feed and treatment times used.

The second set of experiments, with results shown in Fig. 3, focused on maize kernels contaminated in the lab with *A. flavus* spores to simulate natural contamination. These experiments dealt only with the CP and PTG approaches, which were identified as the most effective in the previous set of data, as illustrated in Fig. 2.

A comparison of Figs. 2 and 3 clearly shows that treatments on maize kernels result generally less effective than those on PDA. Additionally, the standard deviations in Fig. 3 suggest that the results for kernels are less reproducible, likely due to the irregular shape of the kernels. Among the treatments tested, the PTG-O_2 20 min appeared to be the least effective, although any significant difference ($P < 0.1$) was detected from statistical analysis. In contrast, for the CP approach, particularly at 3 min treatment time, the CP-O_2 treatment exhibits greater efficacy compared to CP-Air. At 20 min treatment time, the CP processes display similar effectiveness independently on the feed used.

3.2. Activity of the plasma treatments on F. proliferatum

Similarly to *A. flavus*, the conidial suspensions of *F. proliferatum* spread on PDA also showed complete inhibition of conidial germination under all conditions tested in both CP and PTG experiments, as shown in Fig. 4.

Maize kernels contaminated with *F. proliferatum* were exposed to CP and PTG treatments using either O_2 or air-feeds; the resulting inhibition growth data are presented in Fig. 5. As for *A. flavus*, on maize kernels contaminated with *F. proliferatum* the PTW approach proved less effective than the other methods, with significant differences ($P < 0.0001$) in inhibiting the conidial germination, regardless the gas feed. Indeed, the two other approaches yielded approximately 60% inhibition across all treatment times and feeds used, except for the PTG-air 20 min treatment, which gives about 90% inhibition. Similarly to what is found for *A. flavus*, the PTG-air 20 min treatment appears to be the most effective, significantly different ($P < 0.0001$) from the other treatments. Overall, under the experimental conditions investigated, regardless of the gas feed used, the PTW approach results less effective than the two approaches in dry conditions.

We can conclude that for maize kernels artificially contaminated with *F. proliferatum* or *A. flavus*, an inhibition percentage of conidial germination of at least 50% was obtained with the CP and PTG treatments investigated.

3.3. Activity of plasma treatments on mycotoxin contamination

After having established the various efficacy of CP and PTG treatments on conidial germination of *F. proliferatum* and *A. flavus*, we have investigated the ability of the plasma processes to degrade AFB_1 and FB_1 mycotoxins produced by these fungi on filter papers and on maize kernels artificially contaminated (50 μg/kg AFB_1; 1000 μg/kg FB_1). Indeed, as shown in Fig. 6, all treatments exhibit satisfactory degradation rates for AFB_1 on filter paper, higher than 52%, with the exception of the CP-O_2 3 min treatment. In contrast, for FB1 on paper a slightly lower degradation efficacy is measured, higher than 40%, compared to AFB_1. A significant difference between the CP-O_2 20 min and the other treatments is observed for the degradation of FB_1 on paper. Most treatments lead to higher degradation efficiency when applied to a standard mycotoxin solution, except for the CP-O_2 20 min one which shows a higher efficiency of FB1 degradation on maize kernels. Overall, only minor differences in degradation rate are observed in all approaches and

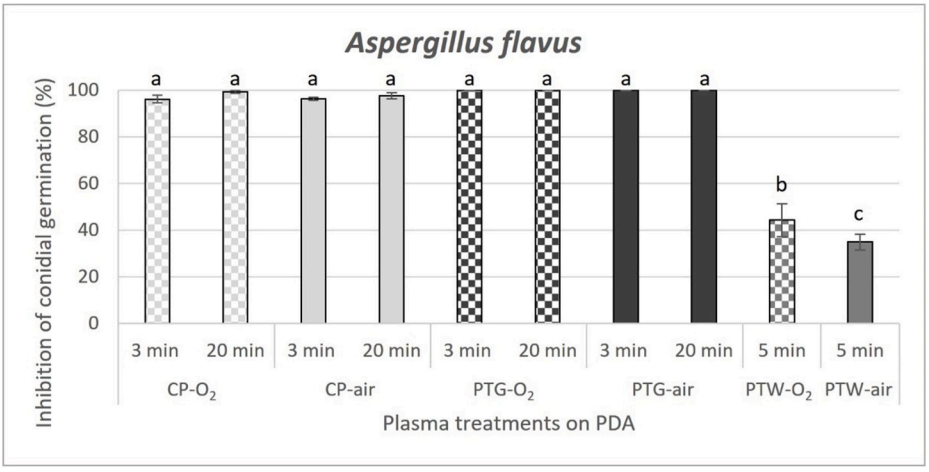


Fig. 2. Inhibition of conidial germination of *Aspergillus flavus* grown on potato dextrose agar (PDA) after plasma treated tap water (PTW), direct plasma treatment (CP) and plasma treated gas (PTG), with air or O_2. Inhibition values were calculated by comparing the germinated conidia of each treatment thesis with the relative control. Standard deviations are also reported. Means with a different letter indicate significant differences between treatments at 0.05 level of significance.

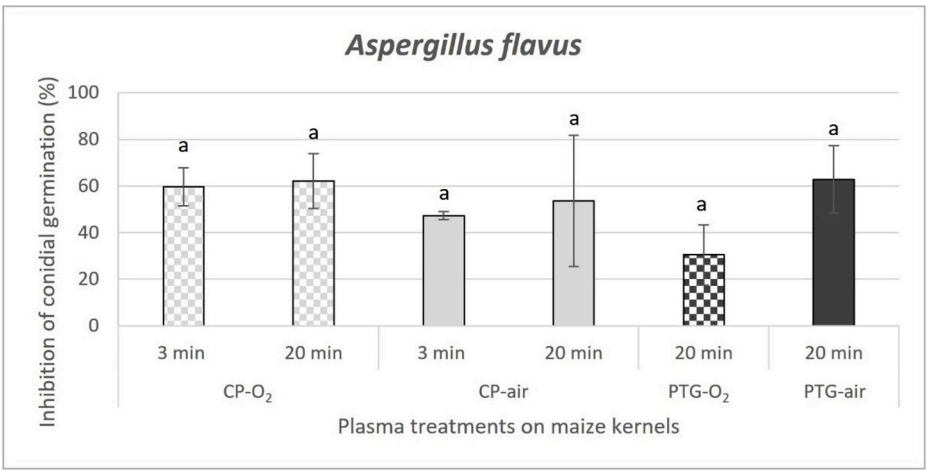


Fig. 3. Inhibition of conidial germination of *Aspergillus flavus* contaminating maize kernels surface, after plasma treated tap water (PTW), direct plasma treatment (CP) and plasma treated gas (PTG), with air or O_2. Inhibition values were calculated by comparing the germinated conidia of each treatment thesis with the relative control. Standard deviations are also reported. Means with a different letter indicate significant differences between treatments at 0.05 level of significance.

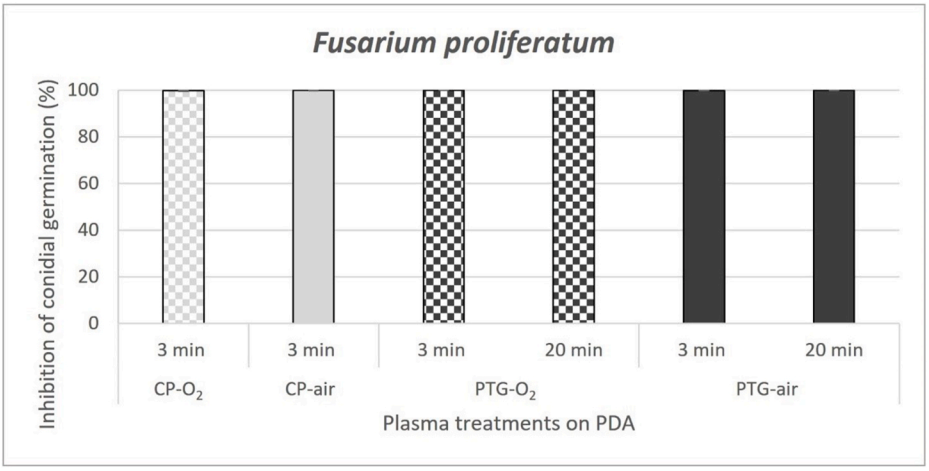


Fig. 4. Inhibition of conidial germination of *Fusarium proliferatum* grown on potato dextrose agar (PDA), after plasma treated tap water (PTW), direct plasma treatment (CP) and plasma treated gas (PTG), with air or O_2. Inhibition values were calculated by comparing the germinated conidia of each treatment thesis with the relative control. Standard deviations are also reported.

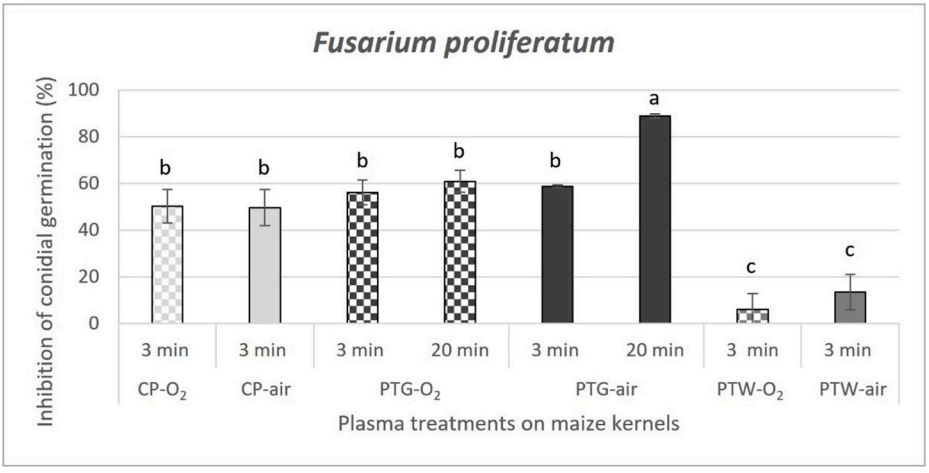


Fig. 5. Inhibition of conidial germination of *Fusarium proliferatum* contaminating maize kernels surface, after plasma treated tap water (PTW), direct plasma treatment (CP) and plasma treated gas (PTG), with air or O_2. Inhibition values were calculated by comparing the germinated conidia of each treatment thesis with the relative control (C). Standard deviations are also reported. Means with a different letter indicate significant differences between treatments at 0.05 level of significance.

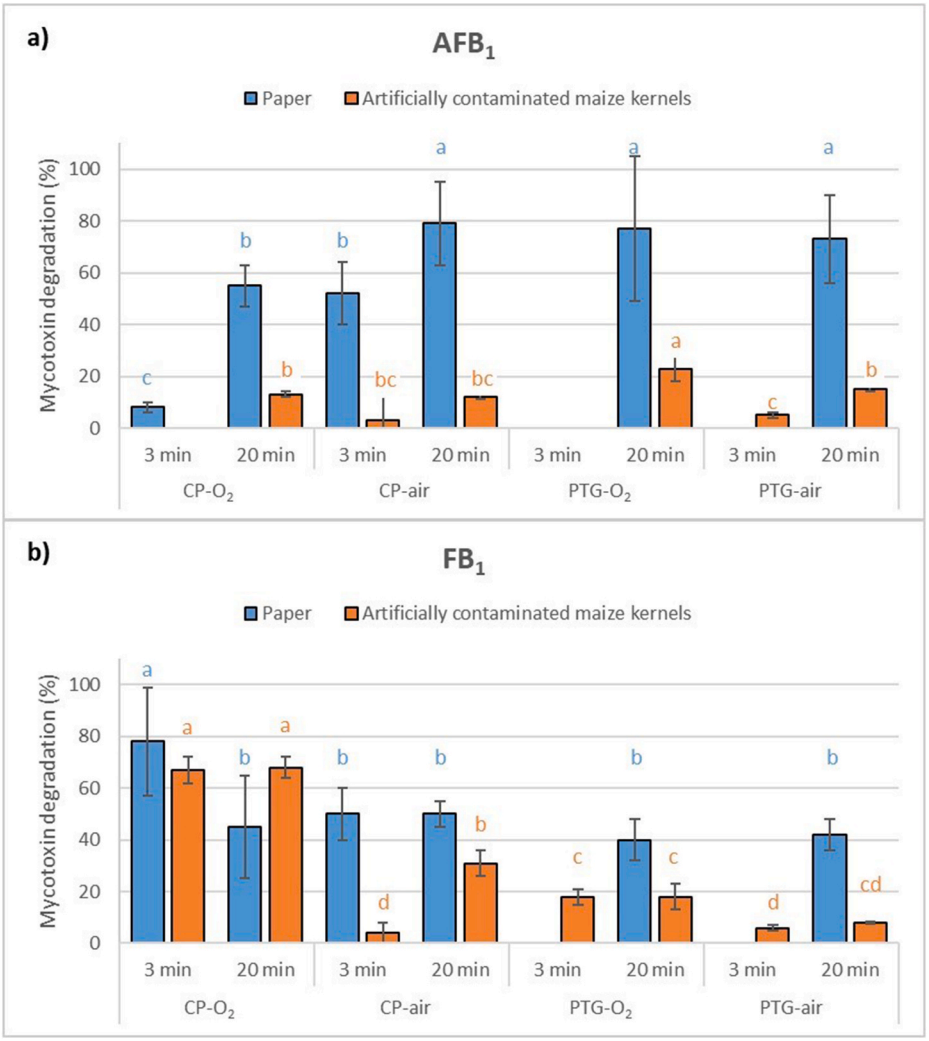


Fig. 6. Degradation percentage (%) of **a)** aflatoxin B_1 (AFB_1) and **b)** fumonisin B_1 (FB_1) on paper and kernel maize after CP or PTG treatments with different feed (O_2 or air) and process time (3 or 20 min). Standard deviations are also reported. Means with a different letter indicate significant differences between treatments at 0.05 level of significance.

feed used.

The degradation percentage of FB_1 after the CP-O_2 20 min treatment was also assessed for maize samples naturally contaminated with 650 ± 56 μg/kg of FB_1, resulting in a 68% contamination reduction, consistent with the detoxification results obtained on paper for the same process.

In summary, for artificially FB_1 contaminated maize kernels, treatments with O_2 were found significantly more effective than those with air, with the CP-O_2 approach showing the highest mycotoxin degradation rate at both treatment times used. Conversely, for AFB_1 contaminated maize kernels, the two indirect PTG processes at the longest treatment time of 20 min gave the best results for AFB_1 degradation.

3.4. Investigation of the degradation products in maize kernels contaminated with AFB_1

Given the effectiveness of the PTG-O_2 20 min treatment against AFB_1 and considering that the conditions of such treatment are particularly gentle, very interesting for practical applications, we decided to investigate the by-products formed after treating AFB1 in such way. The presence of AFB_1 degradation by-products was assessed on maize artificially contaminated and treated in PTG-O_2 20 min mode (Fig. 7).

In Table 2 the proposed degradation products of AFB_1 are listed, with their molecular formula, structures, and n-octanol-water partition coefficient (log P).

To elucidate the structure relative to all detected peaks, MS/MS spectra were analysed (Table 3), and the detected fragments were compared with spectra reported in the literature.

4. Discussion

Food contamination by toxigenic fungi like *Aspergillus*, *Fusarium*, *Alternaria*, and *Penicillium,* as well as by their mycotoxins, is a current health issue arising significant political and economic implications. The CAP technology offers a promising alternative to conventional decontamination methods, demonstrating effectiveness in fungi inactivation, in interfering with mycotoxins biosynthesis, and in mycotoxins degradation (Ganesan et al., 2021; Hamad et al., 2023; Liu et al., 2024).

In this study we compared different plasma assisted approaches, both in dry (CP and PTG) and in wet conditions (PTWS) to assess their efficacy against two toxigenic fungal species and their AFB_1 and FB_1 mycotoxins commonly found in maize. The PTWS approach indeed has been soon excluded after preliminary tests due to its low effectiveness on fungal inactivation in the experimental conditions used in this research.

Our findings indicate that CAP treatments significantly inhibit conidial germination and subsequent colony development of *A. flavus* and *F. proliferatum.* Previous studies have reported similar efficacy of cold plasma processes against mycotoxigenic fungi (Gavahian et al., 2019; Ten Bosch et al., 2017; Veerana et al., 2022). Although studies

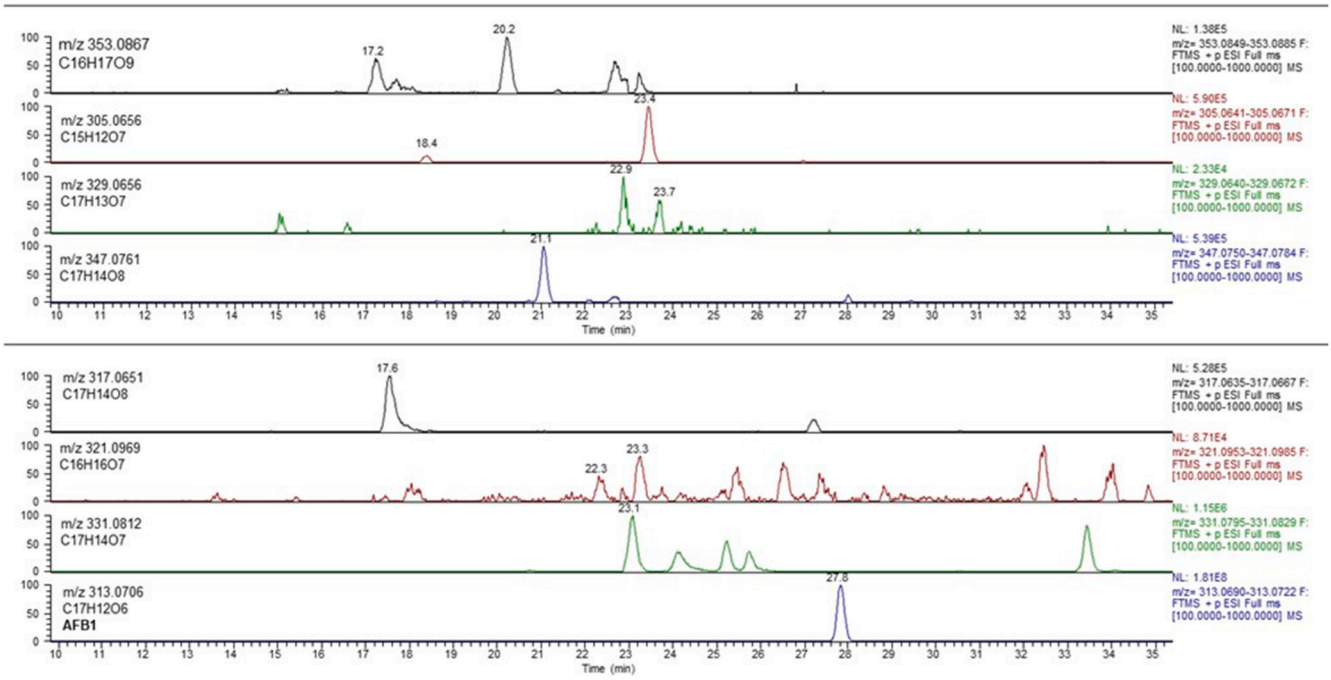


Fig. 7. UHPLC-HRMS chromatogram of maize contaminated with 1 μg/g of AFB_1, treated with the PTG-O_2 20 min process. Peaks attributable to AFB_1 and degradation products (m/z 353.0867, 305.0656, 329.0656, 347.0761, 317.0651, 321.0969, 331.0812 and 313.0706) are shown. Resolution: 70,000 FWHM; extraction window tolerance 5 ppm.

exist on *A. flavus* and aflatoxins reduction after plasma treatments (Devi, Thirumdas, Sarangapani, Deshmukh, & Annapure, 2017; Simoncicova et al., 2018), we believe that our research is the first to examine the effects of CAP processes on *F. proliferatum*, since most of the reports found deal with other *Fusarium* species (Guo et al., 2022; Lo Porto et al., 2019; Wang et al., 2022).

Plasma processes, indeed, were found more effective versus conidia grown *in vitro* on PDA compared to conidia contaminating maize kernels. This discrepancy may be attributed to the complexity of the maize kernels tridimensional shape, which limits the complete exposure of their surface to plasma components (i.e. UV–vis, electromagnetic field etc.) and, mainly, to plasma-generated RONS. Indeed, to improve the reproducibility and the homogeneity of the treatment, the holder of the kernels was shaken during the plasma processes. This could be considered a limitation of the treatments on plant materials but can be easily overcome in industrial applications.

This study, however, shows clearly that fungal development could be inhibited by at least 50% and up to 90% with just few minutes of dry CAP treatments using air or O_2 (Figs. 3 and 5). Notably, in the case of PTG, air plasma appeared more effective than O_2. Working in a closed system we are sure that no contamination from atmospheric N_2 occurs in O_2 plasma processes; this allows to exclude any effect of reactive nitrogen species (RNS) on the targets with respect to reactive oxygen species (ROS). Using O_2 as gas feed of enhances the production of ROS such as ozone (O_3), which is beneficial for reducing microbial load (Zhongping et al., 2019). Higher levels of O_3 in O_2-fed CAPs have been linked to reduced microbial counts on surfaces like hazelnuts (Sen et al., 2019). N_2 is also used as feed for generating reactive nitrogen species that can further interact with O_2 to form gaseous compounds such as NO, NO_2, and NO_3^- ions in water media, all of known antimicrobial activity (Dharini et al., 2023).

The application of various approaches to decontaminate maize kernels indicates that, in the case of CAP treatments, changing treatment time and gas feed among our parameters has minimal impact. The slight effects of the electric field, UV radiation, and both long- and short-lived species may be more influential, instead. Although the CAP process is carried out in remote, the potential influence of other plasma components with RONS cannot be entirely disregarded (Ricciardi et al., 2022). In contrast, for the plasma treatments with the gas effluents from CAPs, PTG, where only chemical effects can be active obviously due to the presence of stable ROS, a clear difference is seen depending on the feed used on maize kernels contaminated with *A. flavus* and *F. proliferatum*. Specifically, the PTG-air process results significantly more effective than the PTG-O_2, highlighting the more active role of RONS (e.g.,NO, NO_2) with respect to ROS (e.g. O_3) in the decontamination process. NO_3^- ions can also be formed in presence of moisture or liquid water.

In contrast, among the approaches tested in our research, PTW treatments cannot be recommended for further investigation, as this approach proved to be the least effective against both *A. flavus* and *F. proliferatum*. The reduced efficacy of PTW compared to CP and PTG treatment may stem from the absence of RONS insoluble in the PTW, such as O_3, which indeed play a crucial role in dry decontamination processes (Bogaerts et al., 2002). Another disadvantage of PTW processes is that their implemention for industrial kernel processing may be more complex, more costly and less versatile than dry decontamination methods.

In terms of mycotoxin abatement, similarly as it is found for conidia contaminated substrates, CAP approaches proved more effective on flat (paper) substrates than on to maize kernels. The reduced degradation of mycotoxins observed in the presence of a food matrix is documented; a recent review (Liu et al., 2024), in fact, highlights that in CAP-based degradation approaches, mycotoxins may not be directly exposed to plasma and its components because hidden in the grooves of the food. Additionally, food features such as surface area, roughness and composition can influence degradation rates. Specifically, roughness and smoothness of the food surface affect the contact area between mycotoxins and reactive species, thereby impacting the degradation efficiency.

Our processes revealed different effects for FB_1 and AFB_1. For FB_1, treatment time did not significantly influence the degradation efficiency. Among the tested approaches, a 20 min CP-O_2 treatment resulted in the highest FB_1 degradation (68%) for artificially contaminated

Table 2
Proposed degradation products with relative molecular formula, exact mass ([M+H]+), retention time (RT) and n-octanol-water partition coefficient (LogP).

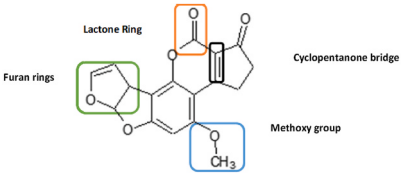
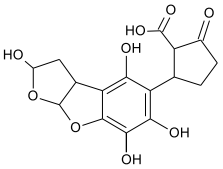
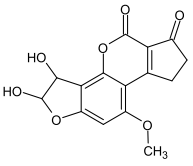
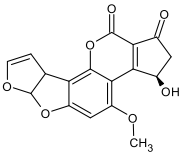
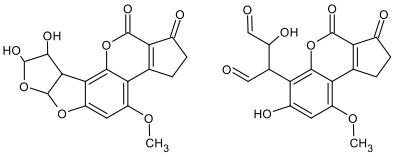
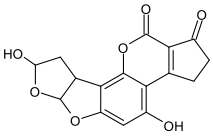
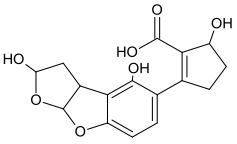
AFB_1 $C_{17}H_{12}O_6$ 313.0706/335.0526 logP:0.45	2,3,6a, 9a – tetrahydro-4-methoxycyclopenta [c]furo [2,3:4,5[furo[2,3-h] chromene-1,11-dione 
Product 1 $C_{16}H_{16}O_9$ 353.0867/375.0686 logP: 2.68 RT: 17.2 min, 20.2 min	2-oxo-5-(2,4,6,7-tetrahydroxy-2,3,3a,8a-tetrahydrofuro[2,3-b]benzofuran-5-yl) cyclopentanecarboxylic acid 
Product 2 $C_{15}H_{12}O_7$ 305.0656 logP: 0.91 RT: 18.4 min	2,3-dihydroxy-9-methoxy-2,3,7,8-tetrahydrocyclopenta[c]furo[2,3-h]chromene-5,6-dione 
Product 3: AFQ1/epi AFQ1 $C_{17}H_{12}O_7$ 329.0656 logP: 0.5 RT: 22.9 min, 23.7 min	3(R) −3 -hydroxy-4-methoxy-2,3,6a, 9a-tetrahydrocyclopenta[c]furo[3′, 2′:4,5]furo[2,3-h] chromene-1,11-dione 
Product 4 $C_{17}H_{14}O_8$ 347.0761/369.0581 logP: 0.39 RT: 21.05 min	(A) AFB_1 8,9 dihydrodiol (B) AFB_1 8,9 dihaldeide 
Product 5 $C_{16}H_{12}O_7$ 317.0656/339.0475 logP: 0.07 RT: 17.6 min, 23.4 min	4,8 - dihydroxy-2,3,6a,8, 9, 9a- hexahydrocyclopenta *[c]*furo[3′,2′:4,5]furo[2,3-h]chromene-1,11-dione 
Product 6 $C_{16}H_{16}O_7$ 321.0969 logP: 0.6 RT: 22.3 min, 23.6 min	2-(2,4-dihydroxy-2,3,3a,8a-tetrahydrofuro[2,3-b] benzofuran-5-yl)-5-hydroxycyclopent-1-ene-1-carboxylic acid 
Product 7	2-(6-methoxy-4-methyl-3a,8a-dihydrofuro[2,3-b] benzofuran-5-yl)-5 oxocyclopent-1-ene-1-carboxyl acid

Table 2 (*continued*)

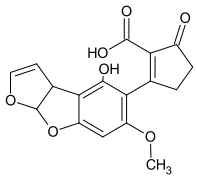
$C_{17}H_{14}O_7$ 331.0812/353.0632 logP: 1.37 RT: 23.1 min	

Table 3
Retention time, molecular formula, and exact mass of the investigated products.

	Parent Ion			Product Ions	
	Retention Time (min)	Molecular Formula	Exact Mass $[M+H]^+$	Exact mass $[M+H]^+$	Molecular formula
Product 1	17.2	$C_{16}H_{16}O_9$	353.0867	No detected	
Product 2	18.4	$C_{15}H_{12}O_7$	305.0656	259.0601	$C_{14}H_{10}O_5$
Product 3 (AFQ1 – epi AFQ1)	22.9/23.7	$C_{17}H_{12}O_7$	329.0656	311.0542	$C_{17}H_{11}O_6$
				283.0588	$C_{16}H_{11}O_8$
				259.0594	$C_{14}H_{11}O_5$
Product 4	21.1	$C_{17}H_{14}O_8$	347.0761	329.0656	$C_{17}H_{13}O_7$
				319.0811	$C_{16}H_{15}O_7$
				311.0538	$C_{17}H_{11}O_6$
				301.0697	$C_{16}H_{13}O_6$
				273.0747	$C_{15}H_{13}O_8$
Product 5	17.6/23.4	$C_{16}H_{12}O_7$	317.0651	289.0707	$C_{15}H_{12}O_6$
Product 6	22.3/23.6	$C_{16}H_{16}O_7$	321.0969	301.0697	$C_{16}H_{13}O_6$
				273.0747	$C_{15}H_{13}O_8$
Product 7	23.1	$C_{17}H_{14}O_7$	331.0812	313.0707	$C_{17}H_{12}O_6$
				285.0757	$C_{16}H_{12}O_5$
AFB_1	27.8	$C_{17}H_{12}O_6$	313.0706	285.0757	$C_{16}H_{12}O_5$
				241.0495	$C_{14}H_9O_3$

maize, a finding that was also confirmed for naturally contaminated kernels.

In contrast, treatment time was particularly relevant for degrading AFB_1, as the maximum degradation (23 ± 5% on kernels and 77 ± 28% on paper) was only achieved after 20 min of the PTG-O_2 process. These results were consistent for both paper and artificially contaminated kernels. The impact of exposure time on AFB_1 degradation was also studied by Siciliano et al. (2016), who assessed the efficiency of DBD plasma for degrading AFs in standard solutions under different conditions. They found that at low plasma power (400 W) 12 min exposure time was needed to be effective, while in more aggressive conditions, 1000 W power with 100% N_2 feed, 100% degradation of AFB_1 was achieved even at the short exposure time of 1 min. When comparing our data (from dry food) with those reported by Siciliano et al. (2016), which were conducted on liquid solutions of mycotoxins, it is important to consider that the liquid matrix can actively contribute to the formation of additional RONS, further aiding mycotoxin degradation.

In artificially contaminated kernels, the PTG-O_2 treatments achieved a higher degradation of AFB_1, comparable with that reported by Iqdiam et al. (2020) for aflatoxin degradation in peanuts after CAP jet treatments. However, our approach is clearly less invasive and quicker, as it can process several seeds simultaneously. It is important to mention that the AFB_1 concentration used in our research on artificially contaminated kernels was ten times higher than the allowed limit.

We have also investigated the degradation products of AFB_1 after a 20 min PTG-O_2. treatment. So far, only a limited number of studies have explored the degradation pathways and products of mycotoxins plasma treated in remote. Most papers on AFB_1 degradation products focus on modifications of the terminal furan rings (Wielogorska et al., 2019); indeed, alterations to the lactone ring and the methoxy group have also been reported (Wang et al., 2015).

AFB_1 can be metabolized and bioactivated by CYP450 enzymes, leading to the formation of the highly toxic epoxy compound aflatoxin B1-8, 9-epoxide, which can bind to intracellular biomacromolecules like DNA, RNA, and proteins, causing gene mutations, thus potentially resulting in cytotoxicity and carcinogenesis (Wang et al., 2023). The difuran ring in AFB_1 is considered primarily responsible for its toxicity and carcinogenicity, particularly the double C=C bond on the terminal furan ring (Shi et al., 2019).

Our study identified the most abundant degradation product in the PTG-O_2 treated maize samples at m/z 305.0656, resulting from the cleavage and hydroxylation of the furan ring. This finding is in agreement with those of Wielogorska et al. (2019). We identified seven AFB_1 degradation products after accurate mass measurements, with an error lower than 2 ppm, and fragmentation pattern analysis, as detailed in Table 3.

Product 3 (m/z: 329.0656) is related to AFQ1 or epi-AFQ1, a metabolic AFB_1 product produced by cytochrome P450 enzymes, which is readily excreted in urine. Product 1 (m/z: 353.0867) results from the addition of a hydroxyl group to the benzene ring. Product 4 (m/z: 347.0761) differs by 34 mass units from AFB_1, suggesting the presence of two hydroxyl groups; it is attributed to AFB_1 8,9-dihydrodiol or AFB_1 dialdehyde, as noted by Loi et al. (2023). Product 5 (m/z: 321.0969) is believed to form through an addition reaction at the C(8)–C(9) bond, accompanied by lactone ring opening and O-cleavage of the methoxy group at C(4) of the benzene ring, a product also reported by Wang et al. (2015). Finally, product 7 (m/z: 331.0812) was detected by Shi et al. (2017) after a high-power CAP treatment of pure AFB_1 powder on a glass slide. The double C=C bond on the terminal furan ring is the main functional group responsible for toxic effects, and our results demonstrate that plasma-generated ROS can react with this moiety of the molecule.

Based on the considerations presented, the plasma-chemical treatments discussed in this paper offer a valid and significantly more versatile alternative to the technologies currently employed and tested for food and seed decontamination. Among the promising low-temperature decontamination methods, high-pressure carbon dioxide (HPCD) could be a potential alternative to plasma-based approaches, particularly due to its ability to operate at lower temperatures than thermal pasteurization (Zulli et al., 2024). However, HPCD has limitations, notably requiring the sample to be moist for optimal efficacy, which makes it less suitable for dehydrated seeds like maize (Garcia-Gonzalez et al., 2007). While HPCD is effective against microorganisms, it has minimal effect on biomolecules such as mycotoxins, as CO_2 exhibits little reactivity with these toxins. In contrast, plasma-assisted technologies offer similar low-temperature benefits but are also effective in degrading mycotoxins, as shown in our study. Moreover, plasma-based methods can be easily integrated into existing production lines and applied after packaging, enhancing their potential for industrial use.

5. Conclusions

This study investigates the feasibility of fungal decontamination and mycotoxins degradation through plasma assisted processes with three different approaches. The results indicate that both *A. flavus* and *F. proliferatum* are significantly impacted by plasma treatments, both *in vitro* and on maize kernels. A notable advantage of the indirect use of CAPs has been registered, with no contact on the kernels, which minimizes possible heating of the target. Our results demonstrate that the chemical effects of plasma on fungi arise from a combination of RONS. Specifically, air-fed plasmas in the PTG configuration result more effective than those fed with O_2. The investigation on mycotoxins shows that both air and O_2-fed CAPs possess high degradation potential for AFB_1, while CP-O_2 treatment proves most effective to degrade FB_1, probably due to structural differences between the molecules.

This paper highlights the significant impact and efficacy of remote plasma-assisted approaches in promoting the decontamination of food from fungi and mycotoxins. It emphasizes the versatility of these soft physical decontamination methods, as they can be consistently applied regardless of the target of interest.

CRediT authorship contribution statement

Stefania Somma: Writing – original draft, Investigation, Data curation, Conceptualization. **Mario Masiello:** Writing – review & editing, Investigation, Data curation, Conceptualization. **Miriam Haidukowski:** Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Biancamaria Ciasca:** Writing – original draft, Validation, Methodology, Investigation, Data curation. **Eloisa Sardella:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Data curation, Conceptualization. **Pietro Favia:** Writing – review & editing. **Fabio Palumbo:** Writing – review & editing, Funding acquisition. **Marianna Roggio:** Writing – review & editing. **Antonio Moretti:** Writing – review & editing, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors have nothing to declare If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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