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# Cold Atmospheric plasma treatment trigger changes in sun-dried tomatoes mycobiota by modifying the spore surface structure and hydrophobicity

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#### 47 Abstract

48 49 The contamination of sun-dried tomatoes during processing can have a decisive impact on the 50 quality of the finished product. In this study we investigated how Cold Atmospheric Plasma (CAP) 51 under high surface power density (SPD) values can reduce fungal contamination in sun-dried 52 tomatoes. In the application of this innovative processing method, the established "regime" for air plasma chemistry was the transition regime or NOx regime. First, we isolated and identified the 53 54 mycobiota present on the tomatoes surfaces by mean of the analysis of the ITS region. The analysis revealed 32 different species, with A. niger, A. tubingensis, A. chevalieri, A. flavus, and A. 55 56 alternata being the most abundant. Then, to reduce the fungal population, CAP-NOx was applied 57 for 5, 10, 20 and 30 min on the surface of dried tomatoes. After incubation for 10 days, we observed that the antifungal effect was species and dose-dependent. In vitro investigation on the most 58 abundant species revealed that A. chevalieri PSJ144 was the most sensitive species (almost 90%) 59 immediately after 5 min of CAP treatment. With the increase of the exposure time up to 30 min, a 60 strong reduction ( $p \le 0.05$ ) of spore germination of *A. alternata* PSJ77 and *A. tubingensis* PSJ100 61 62 was observed (98 and 92%, respectively). However, spores of A. niger PSJ38 and A. flavus PSJ30 63 showed the highest resistance to the treatment. Moreover, the reparameterized Weibull function allowed to obtain useful information about 64 germination kinetics as a function of time of CAP-NOx treatment, revealing that the resistance of 65 the spores was: A. chevalieri<A. alternata<A. tubingensist<A. flavus< A. niger. In situ analyses 66 67 confirmed a significant effect on natural fungal contamination by CAP-NOx treatment (76.5 % of reduction), likely due to cell membrane rupture and cell death caused by plasma radicals. In 68 69 addition, Pearson correlation analysis showed that spore resistance was highly correlated (p=0.98)

- with their hydrophobicity. In a nutshell, our results clearly indicate that CAP-NOx treatment is an
  effective technique to reduce fungal contamination in sun-dried tomatoes. Among non-thermal
  processing methods, CAP shows promising perspectives of application in the tomato industry, to
  mitigate the effects of energy price rises.
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# 80 Introduction

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Daily consumption of fruits and vegetables, in sufficient quantity and quality, helps prevent diseases such as cardiovascular diseases, diabetes and cancer, as well as deficiencies of micronutrients and essential vitamins. The World Health Organization (WHO) ranks inadequate consumption of fruits and vegetables sixth among the 20 risk factors for human mortality. Although these products are known to contain a natural non-pathogenic epiphytic microbiota, they can be contaminated with microorganisms that can be pathogens from human and animals, and can grow during harvesting, transportation, processing, and handling.

89 Tomato (Lycopersicon esculentum L. var. Excell and Aranca) is a highly perishable and fragile 90 vegetable, which is highly susceptible to contamination by microorganisms and mechanical 91 damage during transportation, processing, and storage (Hegazy, 2017). They are not only consumed as fresh produce, but also processed into a variety of products, such as pulp, ketchup, 92 93 sauces, paste, juices, and dried tomatoes (Sanzani et al., 2019). In this context, several strategies are used to produce dried tomatoes. Sun-drying, which is the oldest among all the drying 94 95 techniques, is still one of the most commonly used methods to produce dried tomatoes. Nowadays, 96 sundried tomato is an important ingredient in the food and catering industry, but the quality of this 97 product is not always constant (Sohail et al., 2011). During production, the fruit is sliced to increase 98 the surface-area to volume ratio for the loss of moisture, and the pieces are then dried in open spaces under the sun, where they can come into contact with microorganisms, dirt, soil and insects, 99 100 leading to possible microbial contamination of the product (Canakapalli et al., 2022). Tomato 101 slices are left in full sun for 4–8 days until they have lost most of their moisture content up to 10– 15% (Oberoi et al., 2007). In Italy, dried tomatoes are first cured with sodium chloride for 7 days, 102 103 and then stored at room temperature without humidity control, reaching a shelf-life of around 12 104 months at room temperature. Drying is a critical step in processing, which can lead to fungal 105 contamination from the environment, which can affect the quality of the product (Kakde & Kakde, 106 2012; Sanzani et al., 2019). In addition, rehydration of dried fruit under unsuitable storage conditions may reactivate the fungal growth with subsequent mycotoxin formation (Karaca et al., 107 108 2010). Some studies have reported the presence of fungal spores in dried tomatoes, which belonged 109 to the following species: Aspergillus flavus, Aspergillus niger, Aspergillus parasiticus, Mucor 110 spp., Penicillium brevicopactum, Penicillium chrysogenium, Fusarium culmorum, Aspergillus 111 rugulovalvus formerly Aspergillus rugulosus (syn Emericella rugulosa var. lazuline), A. niger, A. 112 amstelodami, A. tubingensis, A, cristatus, R. oryzae, Cladosporium cladosporioides, Corynascus 113 sepedonium, and Alternaria sp. (Molina-Hernandez et al., 2022; Sanzani et al., 2019; Suleiman et al., 2017). Among these species, Aspergillus ssp., Alternaria sp. and Penicillium sp. deserve 114 115 special attention since several species of these genera can produce mycotoxins. The incidence of 116 these toxic fungal metabolites in dried tomatoes results in economic losses for growers as 117 contaminated export products are rejected (Abdallah et al., 2020; Heperkan et al., 2012). 118 Therefore, the industry today uses strategies aimed to reduce the growth of these molds, which are 119 generally based on the use of chemicals. However, the increasing demand for foods with high quality, with less or no additives, promotes the development of technological alternatives for 120 121 fungal control. In particular, due to the recent rise of energy prices, non-thermal processing 122 methods are appealing options for food manufacturers.

Over the last decades, various non-thermal technologies have been proposed for surface 123 124 decontamination of dried fruits, including gaseous ozone and ozonated water (Zorlugenç et al., 125 2008), ultraviolet (UV–C) alone and in combination with clove essential oils (Gündüz & Korkmaz, 126 2019), pulsed light (PL) (Aguiló-Aguayo et al., 2013), ultrasound (Görgüç et al., 2021), gamma  $(\gamma)$  irradiation (Hamanaka & Chandel, 2009), electron beam irradiation (Mousavi Khaneghah et 127 128 al., 2020), microwave (Popelá rová et al., 2021), and cold atmospheric plasma (CAP) (Lee et al., 129 2015; Molina-Hernandez et al., 2022). The latter technology involves the use of a mixture of ionized gas consisting of charged particles, electric fields, ultraviolet (UV) photons, and reactive 130 131 species, which can exert a strong oxidative power (Laurita et al., 2021). The chemistry that governs 132 the atmosphere inside a plasma reactor depends on the surface power density (SPD), which is the 133 ratio between the power absorbed by the plasma and the surface area of the electrode. According 134 to Simoncelli et al. (2019), two different regimes can be observed below and above a SPD threshold of 0.1 W/cm<sup>2</sup>: 1) small SPD values, where the chemistry is dominated by ozone 135 formation reactions (O<sub>3</sub> –regime); 2) high SPD values, where the formation of NO, N, NO<sub>2</sub>, NO<sub>3</sub>, 136 137 N<sub>2</sub>O<sub>5</sub>, O, O<sub>3</sub> dominates, in a condition that is known as transition regime or NOx regime. Few 138 publications have reported the potential antibacterial activity through the generation of Reactive 139 Nitrogen Species (Wang et al. 2022) by plasma treatment (Shaw et al. 2018). Some researchers 140 have been proposed that NO and the production of intracellular derivatives, such as peroxinitrite 141 and carbonate radicals, can act as effective antimicrobial agents, causing biological effects (DNA

damage, binding to iron centers, oxidiation of thiols, cysteine, etc.) that induce permanent damage

143 in microorganisms (Hao et al. 2014).

144 Recently, we have shown that CAP-O<sub>3</sub> treatments affect the fungal community structure in 145 sundried tomatoes, as fewer fungi were isolated, and their diversity decreased with prolonged CAP 146 treatments (Molina-Hernandez et al., 2022). To the best of our knowledge, there are no studies in 147 the scientific literature on the application of CAP under NOx regime to decontaminate dried fruits. 148 Thus, the aim of this study was to evaluate the effects of CAP at NOx regime on the fungal 149 population on the surface of sundried tomatoes collected in different regions of Italy. First the 150 predominant mycobiota in sundried tomatoes was identified by molecular methods before and after 151 treatment. Next, spore inactivation of the most abundant fungal species, isolated from dried tomato 152 samples, was investigated.

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# 2. Materials and methods

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### 155 *2.1. Samples*

Twenty-three batches of sundried tomatoes (2 kg) were randomly collected from retailers across Abruzzo, Puglia, and Umbria regions in Italy during March 2021. All samples were of commercial quality; samples were selected to avoid visible damage or macroscopic contamination by filamentous fungi. To avoid fungal development without affecting the viability of spores present in sundried tomato, samples were stored in vacuum packed at 20°C until analysis.

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## 2.2 Natural mycobiota in sundried tomatoes

# 163 *2.2.1. Fungal isolation*

For each batch, ten sundried tomato fruits were randomly selected for each treatment and for the untreated control. For the control and after the treatments, each fruit sample was cut into squares of approximately 1.5 x 1.5 cm, and subsequently the different sub-samples were aseptically placed on different culture media: Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Nutrient agar (WL), and Dichloran Glycerol agar base (DG18) for xerophilic filamentous fungi. Chloramphenicol (0.05 g L<sup>-1</sup>), purchased from Liofilchem (Liofilchem, Roseto degli Abruzzi-Italy), was added to all culture media with to prevent bacterial growth. All Petri dishes were 171 incubated for 7 to 10 days at 25°C with 12 h of light and 12 h of darkness in a humid chamber. 172 The plates were then examined to measure the percentage of colonized sub-samples, and fungal 173 diversity (morphotypes). The different colonies were tentatively identified according to their 174 morphology (Samson, Hoekstra and Van Oorshot, 1984) and grouped by their morphological 175 appearance. Pure cultures were obtained from hyphal tip transfer to PDA media and stored at 5°C. 176 The percentage of frequency of each morphotype (MF) for each treatment was calculated 177 according to:

178 MF (%) = ( $\sum$  isolates of the morphotype)/( $\sum$  all fungal isolates) × 100

179 Finally, to increase the rate of fungal growth, the plates were incubated at 30°C.

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# 182 2.2.2. Phenotypical and molecular identification of filamentous fungi

183 To identify the isolates based on the morphological and growth characteristics, single colonies 184 were purified in malt extract agar (MEA) (Liofilchem, Roseto degli Abruzzi-Italy), and 185 subsequently isolated filamentous fungi were identified based on the morphological characteristics 186 under a light microscope according to Munitz et al. (2013). Then, the fungal isolates were 187 tentatively assigned to different genera based on the size and shape of the spores and mycelia. To 188 confirm the identity of the fungi, molecular identification was then carried out according to the 189 method reported by Delgado-Ospina et al. 2021. The PCR assay was performed using the primers 190 listed in Table 1. The ITS region was amplified with the primer pair ITS1-ITS4, ITS1-ITS2. 191 Additional loci (β-tubulin, Calmodulin) were used to identify Aspergillus species. All primers 192 used were purchased from Sigma Aldrich (Saint Louis, Missouri, USA).

193

194 Table 1. Primers used for PCR assay.

Gene name	Gene	Length bp	Primer	Sequences $(5' \rightarrow 3')$	Reference
Internal	ITS (1-4)	420-825	ITS1 (F)	5'TCCGTAGGTGAACCTGCGG3'	
transcribed spacer			ITS4 (R)	5'TCCTCCGCTTATTGATATGC3'	
1 (ITS1) and ITS2	ITS (1-2)	565-613	ITS1 (F)	5' GGAAGTAAAGTCGTAACAAGG 3'	— (Glass &
regions and the			ITS2 (R)	5' TTGGTCCGTGTTTCAAGACG 3'	Donaldson,
5.8S ribosomal			. ,		1995)
DNA (rDNA)					
region					

β-tubulin	ben A	1125	β-tub 2a (F)	5'GGTAACCAAATCGGTGCTTTC 3'	
			β-tub 2b (R)	5'ACCCTCAGTGTAGTGACCCTTGGC 3'	_
Calmodulin	cmdA	543	Cmd5 (F)	5'-CCGAGTACAAGGAGGCCTTC-3'	— (Makhlouf et
			Cmd6 (R)	5'-CCGATAGAGGTCATAACGTGG-3'	— al., 2019)

196	Abbreviation: F: Forward, R: Reverse

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199 2.3. Plasma Treatments

A detailed description of the plasma system used for the treatments has already been given in a 200 201 previous work (Molina-Hernandez et al., 2022), but the most relevant details are reported here for 202 the sake of completeness. Cold Atmospheric Plasma (CAP) was generated by a Surface Dielectric Barrier Discharge (SDBD) placed at the top of a closed chamber, defining a confined atmosphere. 203 204 A high voltage generator produced a sinusoidal waveform with a peak voltage of 6 kV and a 205 repetition frequency of 23 kHz; the power density absorbed by the plasma source was of 425.35±25.79 W, resulting in a surface power density of 2,6 W/cm<sup>2</sup>. Treatments were carried out 206 at room temperature ( $26 \pm 1$  °C). Thirty sundried tomato pieces were placed side and successively 207 treated as described in paragraph 2.2.1. Afterward, the plate with the samples was subjected to 208 209 CAP treatment at 20 cm perpendicularly from the SDBD.

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215 2.4. Optical Absorption Spectroscopy (OAS) measurements

OAS measurements were performed as described by Simoncelli et al. (2019). More specifically,
OAS measurements rely on the Lambert-Beer law, which correlates the amount of light absorbed
by a certain species to the absolute concentration of such species:

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$$n_k = -\frac{1}{L\sigma_k} \ln \frac{l}{l_0}$$

220

where *n* is the concentration of the *k*-th species, L is the optical path (25 cm),  $I_0$  is the intensity of incident light, and *I* is the residual light intensity after the absorption. The wavelengths selected for the absorption measurements performed in this study and the absorption cross-sections of the absorbing species of interest, O<sub>3</sub> and NO<sub>2</sub>, are listed in Table 2. These wavelengths were defined, in accordance with Moiseev et al. (2014), to maximize the absorption of the molecules while minimizing the contribution, and thus the interference from other absorbing molecules.

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**Table 2.** Absorption cross-sections in  $cm^2$  of the species of interest at each selected wavelength.

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Selected	<b>O</b> <sub>3</sub>	NO <sub>2</sub>
wavelength	cross-section	cross-section
253±1.2 nm	(1,12±0.02) E-17	(1.1±0.3) E-20
400±1.2 nm	(1,12±0.08) E-23	(6.4±0.2) E-19

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The setup conditions used to perform the OAS were the same described by Simoncelli et al. (2019). 232 233 Two LEDs were used as the light source, one with maximum emission at 255 nm and the other with maximum emission at 400 nm. The light beam was focused using optical fibers and fused 234 235 silica lens to obtain a parallel beam passing inside the plasma chamber, at a distance of 20 cm from 236 the SDBD. The same distance was maintained between the SDBD and the samples during 237 treatments. The beam was then collected in a 500 mm spectrometer (Acton SP2500i, Princeton 238 Instruments) and spectrally resolved in the UV, VIS and near infrared (NIR) regions. OAS 239 acquisitions were performed using a grating with a resolution of 150 mm-1 and setting a width of 10 µm for the inlet slit of the spectrometer. A photomultiplier tube (PMT-Princeton Instruments 240 241 PD439) connected to a fast oscilloscope (Tektronix MSO46) was used as detector to allow fast acquisitions (time resolution of 40 ms). The PMT amplification factor was kept constant for all 242 acquisitions. Prior to every measurement, the plasma chamber was opened and flushed with air for 243 244 5 minutes to ensure identical initial conditions. Each measurement was repeated 3 times.

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# 246 2.5. Effect of CAP on natural contaminated sundried tomatoes

247 To evaluate fungal inhibition, the most contaminated batches were subjected to decontamination.

- For each batch, ten different tomatoes were subjected to the CAP-NOx treatment (5, 10, 20, 30,
- 249 min) and successively treated as described in paragraph 2.2.1.

# 251 2.6. Effect of CAP on spore germination

For the most frequent species found in the control samples of sundried tomatoes, the in vitro 252 253 resistance/sensibility to CAP-NOx exposure time was determined. For this purpose, the spores of A. niger PSJ38, A. tubingensis PSJ100, A. flavus PSJ30, Aspergillus chevalieri PSJ144 and 254 255 Alternaria alternata PSJ77 were collected according to the method described by Molina-256 Hernandez et al. (2022), standardized at a wavelength of 620 nm to obtain an optical density (OD) of 0.1 AU, which corresponds to  $1.0 \times 10^5$  spores/mL. The different spore suspensions were placed 257 on petri dishes and treated with CAP for 5, 10, 20, 30, 40 and 50 min, respectively. After the 258 259 treatment, an aliquot of 20µL of the spore suspension was inoculated into glass slides with a thin layer of MEA and incubated at 30 °C for 16 h. Untreated spores were considered as controls. Then, 260 261 spore germination was observed with a light microscope. Spores were considered germinated when 262 their germ tube was longer than that of the same spore (Peralta-Ruiz et al., 2020). All the experiments were performed in triplicate, and a total of 200 spores were counted for each sample. 263

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# 265 2.7. Assay of cell surface hydrophobicity

Hydrophobicity (%) = (A1-A2)/A1.

266 The hydrophobicity index of the outermost surface of the spores was determined by microbial 267 adhesion to hydrocarbons, according to the methodology reported by Wang et al. (2017). The spore 268 suspension solutions were washed twice and then resuspended in PBS to an OD600=1. The absorbance of the spore suspension was measured and defined as A1. Subsequently, one milliliter 269 270 of n-butyl alcohol was then added to 1 ml of the cell suspension in a 15-ml falcon tube. After vortexing for 30 s and 3 minutes of incubation, separation occurred. The absorbance of the lower 271 272 aqueous phase was defined as A2. Hydrophobicity is then expressed as percentage calculated using 273 the following equation:

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- **277** *2.7. Spore viability*

To analyze spore viability immediately after CAP treatment, the spores most frequently found in sundried tomatoes were stained with a mixture of CFDA (carboxyfluorescein diacetate) and propidium iodide (PI) according to methodology reported by Molina-Hernandez et al. (2022).

- While the green fluorescent dye CFDA is able to penetrate both intact and damaged cell membranes, the red fluorescent dye PI can only penetrate cells with a significant membrane damage (Molina-Hernandez et al., 2021). 10<sup>4</sup> spore suspension in PBS was treated with CAP-NOx for 5, 10, 20, 30 and 40 min, then stained with both dyes. A Nikon A1R confocal imaging system (Nikon Corp., Tokyo, Japan) was used to observe spore viability.
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### 287 *2.8. Texture analysis of the sundried tomatoes*

- Firmness (F), skin strength (SS) and elasticity (E) of control and treated tomato samples were evaluated using a texture analyzer (TA.HDi 500; Stable Micro Systems, Godalming, UK) according to the method described by Serhat Turgut et al. (2018). Briefly, a Perspex blade (A/LKB) was used for F measurements of tomato samples at the speed of 2 mm/s. For each tomato, two measurements were made. SS and E were determined using a 2-mm cylindrical stainless probe at the speed of 1 mm/s. For each tomato, 3 points were punctured. For each sample, three tomato slices were measured. F and SS were expressed in g. E was expressed in mm.
- 295

# 296 *Statistical analyses*

- For the *in vitro* studies, to evaluate both the CAP effect on fungal spore germination and the reduction in fungal mycelial growth in response to the treatments, data from each sampling point were shown as the mean  $\pm$  SD and statistically analyzed by ANOVA, followed by individual comparisons using Duncan's Multiple Range Test, at p  $\leq$  0.05 and using Pearson correlation as a measure of linear association between treatments.
- 302 To describe the kinetics of spore inactivation due to time of CAP-NOx treatment, the 303 reparametrized Weibull model proposed by De Flaviis & Sacchetti (2022), in the form of a survival 304 function, was fitted. The model was reformulated as follows:

$$F(t) = N_0 \left\{ e^{\left[ -\left(\frac{t}{\beta}\right)^{\frac{e\beta\mu\beta}{N_0}}\right]} \right\}$$
(1)

The Weibull model, extensively used in many applications (e.g., analysis of non-linear survival curves), was chosen because it can fit different shapes of decay (e.g., sigmoidal curves, long-tailed curves, first order decay) and is a very simple and flexible model (van Boekel, 2008). Moreover, 308 the reparametrized Weibull model utilized in this study allows to obtain three meaningful 309 parameters for the interpretation of the kinetics of spore inactivation: i) the initial percentage of 310 germination (N<sub>0</sub>); ii) the failure time ( $\beta$ ), corresponding to the CAP-NOx treatment time when 311 63.2% of the kinetics are reached; iii) the reduction rate at the failure time ( $\mu_{\beta}$ ). The least squares criterion and the "Levenberg-Marquadt" method were used to fit the models and estimate the 312 parameters N0,  $\beta$  and  $\mu_{\beta}$ . The goodness of fit of the models was evaluated considering the R<sup>2</sup>, the 313 314 coefficient of variation of the root means square error (CV(RMSE)) and the Akaike's Information Criterion (AIC). Moreover, the maximum reduction rate  $(\mu_{max})$  and the lag phase  $(\lambda)$ , 315 316 corresponding to the so-called "shoulder effect" were calculated following the equations reported 317 by De Flaviis & Sacchetti (2022):

$$\mu_{max} = \mu_{\beta} e \left(\frac{D}{e}\right)^{D} \tag{2}$$

$$\lambda = \beta [D^{-D} - (D^{-D} - D^{1-D})(e^D)]$$
(3)

**318** Where *D* is:

$$D = \frac{\mu_{\beta} e\beta - N_0}{\mu_{\beta} e\beta} \tag{4}$$

Statistical differences of estimated and calculated parameters among fungal spores were analyzed
by one-way ANOVA and Least Significant Difference (LSD) post-hoc test. Non-linear regressions
were performed using Wolfram Mathematica software (Wolfram Research, Inc.).

322

#### 323 **3.** Results

324 *3.1 Cold Atmospheric Plasma (CAP)* 

The temporal evolution of O<sub>3</sub> and NO<sub>2</sub> was monitored using OAS. Coherently with the high SPD involved in the treatments, no O<sub>3</sub> was detected, whereas a significant NO<sub>2</sub> concentration was measured and was observed to increase with time, as shown in Figure 1.



329

**Figure 1.** Values of NO<sub>2</sub> after different treatment times. The values are the mean of three repetitions. In each panel, data are mean  $\pm$  SD, and statistical significance is specified with letters (\*p  $\leq$  0.05 as determined by paired Student t-test).

# 334 *3.2 Fungal contamination in sundried tomatoes*

In this study, sundried tomatoes samples were tested for the presence and levels of filamentous fungi contaminants. The analysis revealed that all the tomatoes samples were contaminated with spores that germinated and produced proliferous mycelia, although with high variability in the fungal incidence.

339 A total of 220 filamentous fungi were isolated, and 78, representative of all the sundried tomatoes 340 samples, were identified morphologically at a genus level and successively identified at a species 341 level by PCR. The isolates belonged to 20 genera and represented a total of 32 different species (Table S1). Among the fungal species recovered, Psathyrella candolleana and Trametes elegans 342 343 belonged to Basidiomycetes and the other species belonged to Ascomycetes. The relative abundance of the species identified showed that the genus Aspergillus supported 47% (94 isolates) 344 345 of the cultivatable mycobiota, followed by Alternaria (6.8%), Stemphylium (6.41%), Chaetomium (8.1 %), Arthrinium (4.1%), and Penicillium (3.6 %). Other fungal genera such as Rhizopus, 346 347 Byssochlamis, Psathyrella, Trimmatothelopsis, Eutypella, Chrysonilia, *Canariomyces*, 348 Corynoascus, Trametes, Eutypella, Amesia, Aporospora, Ovatospora, Cladosporium, Gymnascella were also present at lower levels ranging from 1.8 % to 2.7 %. 349

As shown in Figure 2, the most frequent and abundant species in sundried tomatoes were *Aspergillus niger* and *Aspergillus tubingensis*, which were present in 6 and 4 out of 24 samples, 352 representing respectively 9.1 % and 7.3 % of all isolated strains, followed by Aspergillus chevalieri 353 (5.9%), Aspergillus flavus (5.9%), Alternaria alternata (5.9%), and Aspergillus fumigatus (4.5%). The other species were isolated less frequently, and their abundance was very low. It should be 354 355 highlighted that some batches showed very low fungal incidence, consisting of single species and 356 in particular of A. chevalieri, A. fumigatus, A. alternata, P. citrinum, A. nidulans, A. cristatus, 357 Eutypella microtheca, and Crysonilia sitophyla (batches 1, 5, 6, 9, 12, 13, 14, 19, 21, and 23, 358 respectively). The batches with low fungal incidence were probably added with high salt 359 concentration during production.

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# 361 *3.3. Effect of CAP-NOx treatments on the spores naturally present in sundried tomatoes*

Different exposure times to CAP-NOx were tested to achieve the highest fungal inactivation on 362 363 the tomatoes surface. In detail, for the most contaminated batches (4, 7, 8 and 20), ten randomly 364 selected tomato samples were exposed to CAP-NOx for 5, 10, 20 and 30 min. Our results showed a higher fungal inactivation by CAP treatment on the smooth surface of tomatoes than on the inner 365 366 face (data not shown), probably due to the lower accessibility of the CAP-NOx species to spores 367 adhering to the rough surfaces of the sundried tomatoes. Some of the fungal species isolated from 368 the control samples were rapidly inactivated and reached undetectable levels after only 10 min of CAP-NOx. For example, in batch 7, we isolated A. flavus, A. chevelieri, A. alternata, A. niger, A. 369 370 tubingensis, Eutypella microtheca, Amesia cymbiformis, but after 5 min of treatment Eutypella 371 microtheca, Amesia cymbiformis, A. chevalalieri and A. tubingensis were no longer found. With the increase of exposure time to 10 min, we isolated only A. alternata, A. flavus and A. niger and 372 373 after 30 minutes of treatment, A. niger was the only isolated species under artificial environmental 374 conditions (95 % HR and 26°C) with a lower incidence than in the control samples. Thus, 30 mins 375 of exposure to CAP-NOx was able to reduce fungal contamination by 76.5 %.



Figure 2. Frequency of the filamentous fungi isolated on the surface of sundried tomatoes belonging todifferent batches. Created with Datawrapper.

#### 384 *3.4. Effect of CAP-NOx time exposure on spore germination*

385 To investigate the inactivation dynamics of CAP-NOx on spore germination in vitro, we 386 considered the spores of selected strains of the species most commonly isolated in the tomatoes 387 batches such as A. alternata PSJ77 A. chevalieri PSJ144, A. tubingensis PSJ100, A. flavus PSJ30, 388 and A. niger PSJ38. In general, the CAP-NOx treatments significantly reduced spore germination 389 but with different exposure times. In fact, spores of A. chevalieri PSJ144 showed a greater 390 reduction (almost 90%) immediately after 5 minutes of CAP treatment. Increasing the exposure 391 time up to 30 minutes, a strong reduction (p  $\leq 0.05$ ) of spore germination was observed in A. 392 alternata PSJ77 and A. tubingensis PSJ100 (98 and 92%, respectively). However, spores of A. 393 niger PSJ38 and A. flavus PSJ30 showed the lowest percentage of non-germinated spores in the 394 same treatment. For these two species, the exposure time was extended to 50 min, and the 395 inhibition of spore germination of A. niger PSJ38 and A. flavus PSJ30 was achieved at 98 and 396 95%, respectively, after 40 minutes of CAP-NOx treatment, thus indicating a major resistance of 397 these two species to CAP-NOx treatments.

398 To study the spore inactivation dynamics, the reparameterized Weibull function was modeled to 399 provide useful species-specific information on germination kinetics as a function of time of CAP-400 NOx treatment. The regressions are shown in Figure 3 and individually plots in figure S1, while 401 their parameters and goodness-of-fit indexes were listed in Table 3. The parameter  $\beta$  proved to be 402 an interesting indicator of spore resistance as it indicates the time required to reduce the initial 403 value (N<sub>0</sub>) to the 63.2%. In this regard, A. *flavus* and A. *niger* showed the highest  $\beta$  time, followed 404 by A. tubigensis and A. alternata. A. chevalieri was the most sensitive spore, since  $\beta$  was reached after only 0.1 minutes of treatment. Generally,  $\mu_{\beta}$  and  $\mu_{max}$  confirmed this trend, showing the 405 406 highest reduction rates in A. chevalieri that resulted the spore inactivated faster by the treatment. 407 It is noteworthy that although A. alternata showed a relatively low failure time ( $\beta$ ), it was also 408 characterized by the lowest reduction rates. Concerning the goodness of fit, the models fitted 409 considerably well as shown by the CV(RMSD) in Table 2, except for A. alternata that fitted 410 slightly worse.

411



**Figure 3**. Kinetics of germination reduction in five different fungal spores, as a function of time of treatment, fitted by the Weibull reparametrized model. Dots indicate real data as means of three replications.

416 The regression parameters were listed in Table 3.

417

418

419 **Table 3**. Estimated and calculated parameters from germination kinetics. Different letters in the same 420 column indicate significant differences (p < 0.05) according to LSD post-hoc test. Goodness of fit indexes 421 were reported as mean  $\pm$  standard deviation.

	Estin	nated param	eters <sup>1</sup>	Calculated par	ameters <sup>2</sup>		Goodness of fit	
	N <sub>0</sub> (%)	μ <sub>β</sub> (%/min)	β (min)	µ <sub>max</sub> (%/min)	λ (min)	R <sup>2</sup>	CV(RMSD)	AIC
Aspergillus flavus	96.5ª	17.8 <sup>ab</sup>	37.2ª	17.9 <sup>a</sup>	33.8ª	$0.999 \pm 0.000$	$3.38\pm0.15$	$43.09\pm0.82$
Aspergillus niger	89.6 <sup>b</sup>	9.2 <sup>ab</sup>	35.3 <sup>b</sup>	9.2 <sup>b</sup>	29.2 <sup>b</sup>	$0.997 \pm 0.000$	$6.55\pm0.33$	$51.91\pm0.54$
Aspergillus tubigensis	95.9ª	8.9 <sup>ab</sup>	25.9°	9.0 <sup>b</sup>	19.1°	$0.999 \pm 0.000$	$3.74\pm0.40$	$40.59 \pm 1.75$
Alternaria alternata	87.5 <sup>b</sup>	2.0 <sup>b</sup>	20.2 <sup>d</sup>	3.3°	0.7 <sup>d</sup>	$0.975\pm0.003$	$21.91 \pm 1.25$	$63.88\pm0.39$
Aspergillus chevalieri	90.3 <sup>b</sup>	152.1ª	0.1 <sup>e</sup>	$\rightarrow \infty^3$	0 <sup>3</sup>	$0.998 \pm 0.000$	$8.65 \pm 1.01$	$36.59 \pm 1.84$

422 <sup>1</sup> Computed by fitting Eq. 1.

423 <sup>2</sup> Computed by using Eq. 2 and 3.

424 <sup>3</sup> Values theoretically assigned, as it is impossible to calculate these parameters when no inflection point is present.

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- 427 *3.5. Changes in spore morphology*

428 Microscopical analyses revealed differences in cell morphology as shown in Figure 4. In this case,

429 a clear loss of spore integrity was observed after only 5 and 30 minutes of CAP-NOx for the spores

430 of A. chevalieri PSJ144 and A. tubingensis PSJ100, respectively. Intense bombardment with NOx

431 radicals in A. chevalieri PSJ144 and A. alternata PSJ77 caused serious lesions on the spore surface,

where the spores appeared perforated. The loss of pigmentation of the spores of *A. niger* PSJ38with increasing exposure time was very interesting, with the color changing from classical black

434 to pale yellow, while in the spores of *A. flavus* PSJ30 the color changed from yellow-green to

- white. In addition, the spore surface appeared very smooth and homogeneous after CAP-NOxtreatment.
- 437 To determine if phenotypic changes were correlated with spore viability, fungal spores subjected 438 to CAP-NOx treatment were examined for viability, using CFDA and PI. Live spores stained green 439 and dead spores stained both red (Fig 5). Membrane permeability detection showed that almost all 440 the spores treated with CAP-NOx were stained red after 30 minutes or longer, suggesting that the 441 cell membrane was unable to maintain its function, which could lead to cell death. These 442 observations were reinforced by the lack of viability in extended CAP-NOx treatment. 443 As observed, the spores of A. chevalieri PSJ144 exhibited a cell membrane damage after 5 min of 444 treatment, which reduced their viability by 96.77%, while the spores of A. tubingensis PSJ100 and A. alternata PSJ77 required more time (30 min) to reach similar values (94.59 and 97.22 445
- respectively). In contrast, the spores of *A. niger* PSJ38 and *A. flavus* PSJ30 were strongly resistant,
- 447 as they required 40 mins treatment to show a loss of viability of 93.33 and 94.12%, respectively.



451 Figure 4. Microscopic visualization of A. chevalieri PSJ144, A. tubingensis PSJ100, A. alternata PSJ77,

*A. flavus* PSJ30 and *A. niger* PSJ38 spores before and after treatment with CAP-NOx. Scale bars, 10 μm.





Figure 5. Confocal laser scanning microscopy analysis of cell viability in *A. chevalieri* PSJ144, *A. tubingensis* PSJ100, *A. alternata* PSJ77, *A. flavus* PSJ30, and *A. niger* PSJ 38 after treatment with CAPNOx. Cells were stained with green fluorescence CFDA (carboxyfluorescein diacetate) and red propidium
iodide (PI) dyes. Bars indicate the percentage of cell live (green) and death (red) spore. Image zoom of
spores indicate a total loss of viability after treatment with CAP-NOx. Scale bar 10 um.

### 463 *3.6. Changes in spore hydrophobicity*

Hojnik et al. (2019) suggested that the resistance of A. flavus to direct exposure to gaseous plasma 464 is due to the surface properties of Aspergillus spp. spores, which have extremely hydrophobic 465 466 properties. For this reason, we measured this property in the spores of the species studied here. As 467 can be seen in Figure 6, the most resistant strains to CAP-NOx treatments, namely A. niger PSJ38 468 and A. flavus PSJ30, had the highest hydrophobicity index compared to the most sensitive strains A. chevalieri PSJ144, A. tubingensis PSJ100, and A. alternata PSJ77. Statistical analysis using 469 470 Pearson correlation showed a high correlation (p=0.98) between hydrophobicity and resistance to 471 CAP-NOx. It is should be emphasized that hydrophobicity was significantly reduced in all strains after only 5 min of exposure; however, in the most resistant strain (A. niger PSJ38), exposure to 472 CAP-NOx promoted a gradual change from the hydrophobic to the hydrophilic state of the spore 473 474 surface.

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478Figure 6. Analysis of the surface hydrophobicity of the A. niger PSJ38 (a); A. flavus PSJ30 (b); A. chevalieri479PSJ144(c); A. tubingensis PSJ100(d), and A. alternata PSJ77(e) after treatment with CAP-NOx. Data were480obtained from three independent experiments. Different letters represent significant differences among the481sample (p < 0.05; Tukey HSD post-hoc test).</td>

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#### 485 3.7. *Effect on the texture of the sundried tomatoes*

Table 4 shows the texture parameters measured in sundried tomatoes after different exposure times. After an exposure time of 5 min, no significant differences were observed in the parameters considered. On the contrary, with increasing treatment times over 10 min, some changes were observed. Compared with the untreated sample, firmness was higher after 10 min but lower after 20 and 30 min. Skin Strength decreased after 20 min, but increased after 30 min. Elasticity was unchanged until the 20 min treatment, and then increased after 30 min.

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**495 Table 4**. Textural values in sundried tomatoes untreated and treated with CAP treatments.

Property	Control	5 min.	10 min.	20 min.	30 min.
Firmness	483.3±133.1ª	426.0±104.9 <sup>a</sup>	606.6±124.8 <sup>b</sup>	252.5±139.8°	426.0±104.9°
Skin Strength	1325.8±460.7 <sup>a</sup>	1307.3±202.3ª	1319.0±353.5ª	823.4±521.5 <sup>b</sup>	1697.5±161.4°
Elasticity	5.7±2.7ª	6.5±1.0ª	6.5±1.6ª	6.1±2ª	7.6±1.8 <sup>b</sup>

497 a,b,c Means in the same row with different superscripts are significantly different (P 0.05).

498 499

#### 500 Discussion

Fungal decontamination is one of the biggest challenges for the food industry during production. 501 502 Some fungal species cause a wide range of diseases affecting numerous economically important 503 host plants, including tomatoes, cereals, potatoes, cabbage, broccoli, carrots, ornamentals, citrus 504 fruits, and apples (Kokaeva et al., 2018). Moreover, fungal contamination occurs not only at harvest or post-harvest, but also during processing. In this context, tomatoes (Solanum 505 506 Lycopersicum L.) are processed into a variety of products such as dried tomatoes, where drying is 507 a critical processing step that can lead to contamination by fungi from the environment, which can 508 affect the quality of the product (Zansani et al., 2019). In fact, the importance of fungal 509 contamination in food products refers not only to the possible degradation activity, but also to the 510 ability of many of them to produce mycotoxins. In this study, we investigated the diversity and 511 occurrence of fungal species in sundried tomatoes from the market, and for the first time, the 512 efficacy of the CAP-NOx regime on the decontamination of fungal spores.

513 As our results from 24 different batches showed, the most frequently occurring fungal genus found

- 514 in sundried tomatoes was *Aspergillus* (47.4%). This result is in agreement with Kalyoncu et al.
- 515 (2005), who described this genus as one of the most important genera isolated in tomatoes and

516 tomato paste. The consequence of the presence of Aspergillus is the possible contamination with 517 mycotoxins, of which the most important are aflatoxins (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>), ochratoxin A, and, at to a lesser extent, fumonisins. In different studies, the presence of fungal species potentially 518 519 producing mycotoxins such as A. flavus (aflatoxin, sterigmatotistin) A. fumigatus (fumitremorgin), 520 A. niger and A. tubingensis (ochratoxin A) was detected in tomatoes, tomato paste, and sundried 521 tomatoes (Han et al., 2019; Kalyoncu et al., 2005; Kokaeva et al., 2018; Sanzani et al., 2019). 522 Other genera that showed high frequency in our batches were Alternaria (6.8%) and Chaetomium 523 (6.3%). The genus Alternaria (A. alternata and A. infectoria), which were present in a significant 524 number of samples (62%), are capable of producing a large number of toxins, including alternariol, 525 alternariol monomethylether, altenuene, tenoxin and tenuazonic acid. A. alternata is a saprophytic 526 pathogen of tomato, a necrotrophic latent fungus that causes black spots on the surface of ripening 527 tomatoes, resulting in frequent postharvest losses (Encinas-Basurto et al., 2017). On the other 528 hand, Chaetomium with the species Chaetomium cochliodes and Chaetomium acropollum, are globally ubiquitous fungi found in soil and degraded cellulosic materials (Salo et al., 2020). 529

530 The presence of potentially mycotoxigenic fungi in sundried tomatoes makes it important to adopt 531 systems for disinfection and to minimize their impact on the quality of the treated product. Our 532 results demonstrate that CAP-NOx regime can be a promising strategy to decontaminate the surface of sundried tomatoes from fungal spores. In fact, the reactive species generated by CAP 533 534 under NOx regime compromise to a great extent the integrity of cells (Misra et al., 2011). In our 535 study, the NOx regime was achieved by applying a sufficiently high surface power density to the plasma discharge. Indeed, NOx production with cold atmospheric plasmas in air is known to be a 536 threshold process guided by the SPD by controlling the excitation and dissociation of N<sub>2</sub> molecules 537 538 (Simoncelli et al., 2019). Once these molecules are sufficiently excited, their reaction with O<sub>2</sub> and O<sub>3</sub> molecules leads to the formation of a complex NOx chemistry, including NO, N, NO<sub>2</sub>, NO<sub>3</sub>, 539 540 N<sub>2</sub>O<sub>5</sub> (Hojnik et al., 2019; Molina-Hernandez et al., 2022).

Although the antifungal activity of CAP-O<sub>3</sub> has been demonstrated by several authors (Ambrico et al., 2020; Kang et al., 2014; Molina-Hernandez et al., 2022; Panngom et al., 2014), studies on the antifungal activity of CAP-NOx are lacking. Our data support the hypothesis that exposure of tomatoes to CAP-NOx may be associated with the decontamination of fungal spores on the surface of sundried tomatoes, which depends on the CAP-NOx exposure time, and the characteristics of the target fungi. The results of our *in-situ* study pointed out the efficacy of the treatment in terms of spore inactivation in naturally contaminated tomatoes, which increased with increasing exposure time to CAP-NOx. In fact, we observed a reduction of fungal contamination of 76.5 % after 30 mins of exposure to CAP-NOx. In this regard, the spores of *A. flavus* PSJ30 and *A. niger* PSJ38 were the most resistant after 30 min treatment, resulting in a high survival of these fungi compared to other fungal species found in the tomatoes batches here analyzed. These results indicate that the increased levels of reactive nitrogen species (RNS) following plasma treatment likely played a role in spore inactivation.

554 The antifungal effect of NO has been previously reported (Weller et al., 2001). However, the 555 comparison of the efficacy of the CAP treatment with previous studies is very difficult because it 556 depends on the generator device, voltage, exposure time, initial microbial density, process gas, 557 working distance, and plasma exposure. Wang et al. (2022) reported 96.84% loss of cell viability 558 and membrane integrity of Fusarium spp. after 3-min of CAP using a dielectric barrier surface 559 micro-discharge (SMD) plasma generator. Jo et al. (2014) observed 92% fungal colony forming units (CFU) inhibition of G Gibberella fujikuroi on the rice seed surface, after 120 s of exposure. 560 Our results are in agreement with those obtained by Ambrico et al. (2020), who reported that the 561 562 inactivation of the fungal growth is species dependent. In fact, they observed different survival 563 rates of spores belonging from different fungal species (Botrytis cinerea, Monilinia fructicola, 564 Aspergillus carbonarius and Alternaria alternata) exposed to SDBD plasma. It is important to 565 highlight that inhibition of spore germination, reduction of viability and morphological alterations 566 of cell surface up to spore destruction of these species, demanded diverse treatment times. Moreover, Pańka et al. (2022) found diverse efficacy of Cold plasma (DBD) treatments on 567 568 different fungal spores present in seeds, by using 400 W power and 15 s of exposure time. In this 569 case, the treatment was very effective against Alternaria, Aspergillus, Colletotrichum, Fusarium, 570 *Penicillium* genera, being *Aspergillus ochraceus* the most resistant species. For more intense direct DBD systems, a notable study using an air direct DBD on agar plates achieved significant 571 572 reductions against fungi (1.7 and 1.0 log CFU reductions of Aspergillus oryzae and Alternaria 573 conidia, respectively, within 10 min) (Julák et al., 2018).

- 574 In our study, the specific effect of CAP-NOx treatment on fungal spores was confirmed *in vitro*,
- 575 demonstrating that the spores of the species studied here were resistant to CAP-NOx in the
- 576 following order: A. chevalieri PSJ144 < A. alternata PSJ77 < A. tubingensis PSJ100 < A. flavus
- 577 PSJ30 < A. niger PSJ38. Interestingly, although A. alternata showed a relatively low failure time

578 ( $\beta$ ), it was also characterized by the lowest rates, suggesting that a small group of cell spores could 579 survive a longer treatment time. This may be because *Alternaria* spp. is the only multicellular 580 conidia studied in this work, characterized by a primary cell wall that is melanized and a secondary 581 wall that is not melanized (Ambrico et al., 2020), which may have resulted in a different response 582 to CAP-NOx treatment.

583 Fungal spore survival in the harsh environment of plasma treatment generally depends on: 1) the 584 spore cell wall, which helps prevent radiation damage to DNA, 2) the type of gas used and the gas 585 flow rate, 3) the relative humidity, and 4) the substrate. Fungal spores are very dense and compact 586 structures surrounded by different layers. In particular, the outer layer of most asexual fungal 587 spores is composed of polysaccharides (chitin and combination of a-glucans and b-glucans), and 588 is surrounded by a rodlet layer with a complex structure composed of a phenolic compound 589 (melanin) and a hydrophobic protein (hydrophin) (El Enshasy, 2022). These compounds form a 590 well-structured monolayer in which a combination of neutral, hydrophilic and hydrophobic amino acids are present and which exhibits a uniform hydrophobicity on the outside of the cell (Moonjely 591 592 et al., 2018; Wu et al., 2017). Ambrico et al. (2020) observed that RNS have a direct effect on 593 cells, and especially on the outermost layer, the polysaccharide-rich wall. Other authors suggested 594 that the radicals produced during CAP may oxidize the protein of the spore envelope, leading to a 595 loss of the envelope integrity and therefore making the spore more vulnerable to attack by the 596 radicals generated by plasma (Devi et al., 2017). In fact, bombardment with reactive plasma 597 species creates active sites on the surface of the protein, and the RNS introduced by plasma into 598 the protein structure can also act as quenchers (Bußler et al., 2015). In addition, the accumulation 599 of charged particles on the surface of spores and electrostatic forces can lead to rupture of the cell 600 membrane and subsequently cause cell death (Laroussi et al., 2003; Mendis et al., 2000). In a study 601 conducted by Hojnik et al. (2019) on spores of A. flavus treated with both direct gaseous plasma 602 treatment and indirect treatment with plasma activated aqueous broth (PAB), it was found that 603 direct treatment was more effective than PAB, and this result was attributed to the hydrophobic 604 surface properties of the spores, which make them much more resistant to RONS in the liquid 605 phase. Herein, we found that the survival of spores of the 5 different strains studied to CAP-NOx 606 was highly dependent on spore hydrophobicity. In fact, the spores of A. niger PSJ38 and A. flavus 607 PSJ30, which turned out to be the most hydrophobic ones, were also more resistant to plasma 608 treatments than A. chevalieri PSJ144 spores, which were less hydrophobic. In this respect, spore 609 hydrophobicity might have contributed to the spore resistance, due to the lower interaction 610 between NOx radicals and the small secreted amphipathic proteins called hydrophobins, which can self-assemble into a monolayer that exhibits a uniform hydrophobicity on the cell exterior 611 612 (Moonjely et al., 2018). In particular, the amino acids that form the hydrophobins of A. chevalieri 613 PSJ144 might be more polar and therefore more easily targeted by radical species than less polar 614 ones. In addition, it is important to underline that after each CAP-NOx treatment the spores lost 615 their hydrophobicity, which suggests a direct or an indirect effect of CAP on the protein 616 architecture of the cell wall, leading to a reduction in cell vitality. The degradation of cellular 617 proteins by CAP is poorly described in literature and should be investigated.

618 Another aspect to consider in spore resistance to CAP-NOx is the presence of melanin, a pigment known to contribute to the rigidity of spore cell walls, protecting the cell from stressors such as 619 620 temperature, UV-radiation, and reactive oxygen species (Ott et al., 2021). In this respect, our data 621 support the observations of Ambrico et al. (2020), who found that the darkest-colored spores of A. carbonarius and A. Alternaria showed higher resistance to the treatment compared to the lighter 622 623 pigmented and thinner walled spores of *B. cinerea* and *M. fructicola*. In fact, in our study the spores of A. niger PSJ38 and A. alternata PSJ77 were more resistant to CAP-NOx than those of A. 624 625 chevalieri PSJ144, which showed a 98% inactivation after 5 min of plasma exposure. It should be 626 emphasized that melanin pigments quench reactive oxygen and nitrogen species, and therefore 627 spores depleted of this pigment are more susceptible to ROS and RNS (Ott et al., 2021). This may 628 be the case for A. niger PSJ38, which was depigmented after 40 min of treatment and showed an increasing percentage of spore inactivation. Pal et al. (2014) also found that loss of melanin was 629 630 associated with increased susceptibility of Aspergillus spp. to damage by reactive oxygen species. 631 Therefore, we can hypothesize that proteins of the wall are one of the first targets for biologically 632 active agents generated by CAP-NOx, and that the efficacy of plasma in spore inactivation depends 633 not only on spore hydrophobicity, but also on the presence of melanin that could mitigate the 634 effects of CAP-NOx on spore survival.

Textural properties are important characteristics that determine the product quality, as they
strongly influence consumer's perception. The observed changes in the considered parameters
occurred after 10 min of treatment but did not show a clear trend with respect to treatment duration.
The texture of sun-dried tomatoes is very complex, and, to our knowledge, there are no reports on
the effects of CAP on the structural properties of these products, so it is very difficult to draw

640 conclusions. To understand whether the observed changes cause significant effects on dried tomato641 quality, the texture should probably be evaluated by a panel test.

642 Compared with other methods for reducing fungal growth, CAP-NOx is a fast technology that can 643 decontaminate foods and does not leave toxic residues or post-processing exhaust gases. In 644 addition, it represents a non-thermal alternative for food decontamination, which can be considered 645 appealing in view of the recent rise of energy prices. Moreover, this method is certainly 646 sustainable, because it avoids the impact of chemicals on both the product and the environment. 647 Thus, CAP-NOx could be an attractive approach for producing high-quality tomato products with 648 an extended the shelf-life, with interesting potential effects on the market, considering the 649 possibility to enlarge the trade, differentiate the range of commercialized products, reduce the 650 waste and the cost of the discharge, with economic and social advantages. Furthermore, post-651 harvest treatments with chemicals involve costs for controlling residues, with a direct impact on 652 the price of the finished product (Hernández-Torres et al., 2022).

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#### 655 CONCLUSIONS

656 This study is a first attempt to apply CAP-NOx for treating a dried fruit. The results presented in 657 this paper show for the first time the inhibitory activity of CAP-NOx species against the spores of 658 the most frequent species found in sundried tomatoes, and namely A. alternata, A. chevalieri, A. 659 tubingensis, A. flavus and A. niger. We demonstrated that CAP treatment can effectively reduce 660 the survival rate (76.5%) of fungi on the surface of sundried tomatoes. The Weibull 661 reparameterized model proposed by De Flaviis & Sacchetti (2022) gave useful information on the species-specific inactivation kinetics after CAP-NOx treatment, which involves a differential 662 663 germination of the fungal spores associated with sundried tomatoes. Although four of the tested 664 Aspergillus species have a similar asexual morphological type, they perform differently under the nitrosative stress induced by CAP-NOx. This behavior may be correlated with spore 665 666 hydrophobicity, which was major in the most resistant spores as A. niger. However, more research needs to be done to better explain the vector of environmental adaptations in A. niger against CAP-667 668 NOx. In conclusion, the results of this study open up new perspectives on the potential application 669 of CAP-NOx for fungal decontamination of dried foods.

#### 671 CRediT authorship contribution statement

Junior Bernardo Molina-Hernandez: Conceptualization, Methodology, Software, Validation,
Formal analysis, Investigation, Writing – original draft, Silvia Tappi: Methodology, Formal
analysis, Writing – review & editing; Matteo Gherardi: Methodology, Formal analysis, Writing
-review & editing; Riccardo de Flaviis: Methodology, Data curation, Jessica Laika:
Investigation, Formal analysis, Yeimmy Yolima Peralta-Ruiz: Investigation, review & editing,
Antonello Paparella: Supervision, Writing – review & editing: Clemencia Chaves-López:
Conceptualization, Supervision, Writing – original draft, Project administration.

# 681 Declaration of Conflict of Interest

- 682 The authors declare no conflict of interest

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- A. niger, A. tubingensis, were most abundant species in dried tomatoes.
- CAP reduced the fungal contamination on the dried tomatoes by 76.5 %.
- *A. niger* and *A. flavus* spores were more resistant ones to CAP.
- *A. chevalieri* spores were inactivated after 0.1 min of CAP-NOx.
- Spore hydrophobicity was correlated with the spore resistant to CAP.

# **Declarations of interest**

The authors of the present paper "Cold Atmospheric plasma treatment trigger changes in Sundried tomatoes mycobiota by inducing changes spore surface structure and hydrophobicity of *Aspergillus* species", Junior Bernardo Molina-Hernandez, Silvia Tappi, Matteo Gherardi, Riccardo de Flaviis, Jessica Laika, Yeimmy Yolima Peralta-Ruiz, Antonello Paparella and Clemencia Chaves-López **don't have any conflict of interest** 

# **CRediT** authorship contribution statement

Junior Bernardo Molina-Hernandez: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing – original draft, Silvia Tappi: Methodology, Formal analysis, Writing – review & editing; Matteo Gherardi: Methodology, Formal analysis, Writing –review & editing; Riccardo de Flaviis: Methodology, Data curation, Jessica Laika: Investigation, Formal analysis, Yeimmy Yolima Peralta-Ruiz: Investigation, review & editing, Antonello Paparella: Supervision, Writing – review & editing: Clemencia Chaves-López: Conceptualization, Supervision, Writing – original draft, Project administration. Table 1. Primers used for PCR assay

**Table 2.** Absorption cross-sections in cm<sup>2</sup> of the species of interest at each selected wavelength.

**Table 3**. Estimated and calculated parameters from germination kinetics. Different letters in the same column indicate significant differences (p < 0.05) according to LSD post-hoc test. Goodness of fit indexes were reported as mean  $\pm$  standard deviation.

**Table 4.** Textural values in sundried tomatoes untreated and treated with CAP treatments.

**Table S1**. Identification of the fungi isolates form dry tomatoes, determined by amplifying Internal transcribed spacer 1 (ITS1) and ITS2 regions and the 5.8S ribosomal DNA (rDNA) region,  $\beta$ -tubulin and Calmodulin gene and nucleotide sequences.

Figure 1. Values of NO<sub>2</sub> after different treatment times. The values are the mean of three repetitions. In each panel, data are mean  $\pm$  SD, and statistical significance is specified with letters (\*p  $\leq$  0.05 as determined by paired Student t-test).

Figure 2. Frequency of the filamentous fungi isolated on the surface of sundried tomatoes belonging to different batches. Created with Datawrapper.

**Figure 3.** Kinetics of germination reduction in five different fungal spores, as a function of time of treatment, fitted by the Weibull reparametrized model. Dots indicate real data as means of three replications. The regression parameters were listed in Table 3.

**Figure 4.** Microscopic visualization of *A. chevalieri* PSJ144, *A. tubingensis* PSJ100, *A. alternata* PSJ77, A. flavus PSJ30 and *A. niger* PSJ38 spores before and after treatment with CAP-NOx. Scale bars, 10 µm.

**Figure 5**. Confocal laser scanning microscopy analysis of cell viability in *A. chevalieri* PSJ144, *A. tubingensis* PSJ100, *A. alternata* PSJ77, *A. flavus* PSJ30, and *A. niger* PSJ 38 after treatment with CAP-NOx. Cells were stained with green fluorescence CFDA (carboxyfluorescein diacetate) and red propidium iodide (PI) dyes. Bars indicate the percentage of cell live (green) and death (red) spore. Image zoom of spores indicate a total loss of viability after treatment with CAP-NOx. Scale bar 10 um.

**Figure 6.** Analysis of the surface hydrophobicity of the *A. niger* PSJ38 (a); *A. flavus* PSJ30 (b); *A. chevalieri* PSJ144(c); *A. tubingensis* PSJ100(d), and *A. alternata* PSJ77(e) after treatment with CAP-NOx. Data were obtained from three independent experiments. Different letters represent significant differences among the sample (p < 0.05; Tukey HSD post-hoc test).

**Figure S1**. Individually plots of germination kinetics in five different fungal spores for three replicates fitted by the Weibull reparametrized model. Dots indicate real data. X axis and Y axis indicate time of treatment and percentage of germination respectively.











Click here to access/download;Figure;Figure 5.pdf ±



40 min.

E

40 min.

א. flavus



Figure

Gene name	Gene	Length bp	Primer	Sequences $(5' \rightarrow 3')$	Reference
Internal	ITS (1-4)	420-825	ITS1 (F)	5'TCCGTAGGTGAACCTGCGG3'	
1 (ITS1) and ITS2			ITS4 (R)	5'TCCTCCGCTTATTGATATGC3'	(Glass &
regions and the 5.8S ribosomal	ITS (1-2)	565-613	ITS1 (F)	5' GGAAGTAAAGTCGTAACAAGG 3'	<ul> <li>Donaldson, 1995)</li> </ul>
DNA (rDNA) region			ITS2 (R)	5' TTGGTCCGTGTTTCAAGACG 3'	
β-tubulin	ben A	1125	β-tub 2a (F)	5'GGTAACCAAATCGGTGCTTTC 3'	
			β-tub 2b (R)	5'ACCCTCAGTGTAGTGACCCTTGGC 3'	(Makhlouf et
Calmodulin	cmdA	543	Cmd5 (F)	5'-CCGAGTACAAGGAGGCCTTC-3'	— al., 2019)
			Cmd6 (R)	5'-CCGATAGAGGTCATAACGTGG-3'	

# Table 1. Primers used for PCR assay.

Abbreviation: F: Forward, R: Reverse

Selected wavelength	O3 cross-section	NO <sub>2</sub> cross-section
253±1.2 nm	(1.12±0.02)E-17	(1.1±0.3)E-20
400±1.2 nm	(1.12±0.08)E-23	(6.4±0.2)E-19

Table 2. Absorption cross-sections in  $cm^2$  of the species of interest at each selected wavelength.

 $40.59 \pm 1.75$ 

 $63.88\pm0.39$ 

 $36.59 \pm 1.84$ 

		1011.						
	Estir	Estimated parameters <sup>1</sup>		Calculated parameter <sup>2</sup>		Goodness of fit		
	N <sub>0</sub> (%)	μ <sub>β</sub> (%/min)	β (min)	$\substack{\mu_{max} \\ (\%/min)}$	λ (min)	R <sup>2</sup>	CV(RMSD)	AIC
Aspergillus flavus	96.5ª	17.8 <sup>ab</sup>	37.2ª	17.9ª	33.8ª	$0.999 \pm 0.000$	$3.38 \pm 0.15$	$43.09\pm0.82$
Aspergillus niger	89.6 <sup>b</sup>	9.2 <sup>ab</sup>	35.3 <sup>b</sup>	9.2 <sup>b</sup>	29.2 <sup>b</sup>	$0.997 \pm 0.000$	$6.55\pm0.33$	$51.91\pm0.54$

9.0<sup>b</sup>

3.3°

 $\rightarrow \infty^3$ 

**Table 3**. Estimated and calculated parameters from germination kinetics. Different letters in the same column indicate significant differences (p < 0.05) according to LSD post-hoc test. Goodness of fit indexes was reported as mean  $\pm$  standard deviation.

<sup>1</sup> Computed by fitting Eq. 1.

Aspergillus tubigensis

Alternaria alternata

Aspergillus chevalieri

<sup>2</sup> Computed by using Eq. 2 and 3.

8.9<sup>ab</sup>

 $2.0^{\text{b}}$ 

152.1ª

25.9°

20.2<sup>d</sup>

0.1°

95.9ª

87.5<sup>b</sup>

90.3<sup>b</sup>

<sup>3</sup> Values theoretically assigned, since it is impossible to calculate these parameters when no inflection point is present.

19.1°

0.7<sup>d</sup>

 $0^3$ 

 $0.999 \pm 0.000$ 

 $0.975\pm0.003$ 

 $0.998 \pm 0.000$ 

 $\phantom{-}3.74 \pm 0.40\phantom{0}$ 

 $21.91 \pm 1.25$ 

 $8.65 \pm 1.01$ 

Property	Control	5 min.	10 min.	20 min.	<b>30 min.</b>
Firmness	483.3±133.1ª	426.0±104.9 <sup>a</sup>	606.6±124.8 <sup>b</sup>	252.5±139.8°	426.0±104.9°
Skin	1325.8±460.7 <sup>a</sup>	1307.3±202.3ª	1319.0±353.5 <sup>a</sup>	823.4±521.5 <sup>b</sup>	1697.5±161.4°
Strenght					
Elasticity	5.7±2.7ª	$6.5 \pm 1.0^{a}$	6.5±1.6ª	6.1±2ª	7.6±1.8 <sup>b</sup>

Table 4. Textural values in sundried tomatoes untreated and treated with CAP treatments.

a,b,c Means in the same row with different superscripts are significantly different (P 0.05).

Identity Sample **Closed relative** Primer Accession % number PSJ 77 ITS1-ITS4 100 KF881759.1 **PSJ 65** Alternaria alternata ITS1-ITS4 100 KF881759.1 **PSJ 79** KF881759.1 ITS1-ITS4 100 **PSJ 70** ITS1-ITS4 100 MK226292.1 PSJ 70-1 Alternaria infectoria ITS1-ITS4 100 MK226292.1 **PSJ 74** Amesia cymbiformis ITS1-ITS4 100 MH861721.1 **PSJ 72** Aporospora terricola ITS1-ITS4 99 AF049088.1 99,7 VJT-20 Aspergillus amstelodami  $\beta t 2a$ - $\beta t 2b$ FR775356.2 PSJ-76-1 ITS1-ITS4 100 MT316337.1 **PSJ 79** ITS1-ITS4 100 MT316337.1 PSJ 132-1 ITS1-ITS4 100 MN174037.1 **PSJ 150** Aspergillus chevalieri MN174037.1 ITS1-ITS4 100 **PSJ 131** ITS1-ITS4 MN174037.1 100 PSJ 144 ITS1-ITS4 99 MT316339.1 VJT-2 Aspergillus cristatus ITS1-ITS4 99.32 KY828916.2 ITS1-ITS2 99 JX501356.1 PSJ 30 PSJ 14 ITS1-ITS4 99 MT645322.1 **PSJ 106** Aspergillus flavus 100 ITS1-ITS4 MT558941.1 **PS154** ITS1-ITS4 100 MT292809.1 PSJ 40-1 99 ITS1-ITS4 MF379664.1 PSJ 9 ITS1-ITS4 100 MK841416.1 Aspergillus fumigatus **PSJ 10** ITS1-ITS4 99 MT487775.1 **PSJ 50** OK067466.1 ITS1-ITS4 100 Aspergillus nidulans **PSJ 48** ITS1-ITS4 100 MK397763.1 PSJ 105 ITS1-ITS4 99 MT316339.1 VJT 7 CMD5-CMD6 99,6 HQ632731.1 **VJT 14** βt2a-βt2b 99,7 MN907662.1 **VJT 12** ITS1-ITS4 99,1 MN493772.1 **VJT 28** 99,1 ITS1-ITS4 LC577101.1 **VJT 15** 99,7 JX545078.1 βt2a-βt2b VJT 18 ITS1-ITS4 99.1 MTI23512.1 VJT 1 βt2a-βt2b 99,12 MT597823.1 VJT 6 Aspergillus niger βt2a-βt2b 98,8 KY990205.1 VJT 17 βt2a-βt2b 99,7 KU865178.1 **VJT 26** ITS1-ITS4 98 MK138359.1 **PSJ 38** 99.1 βt2a-βt2b KJ36066.1 CMD5-CMD6 99.1 MH447369.1 Aspergillus rugulovalvus VJT-5 βt2a-βt2b 100 AB248319.1 CMD5-CMD6 **PSJ 100** 100 MK166185.1 **PSJ 109** CMD5-CMD6 100 KY612372.1 **VJT 10** CMD5-CMD6 98,9 KY612372.1 **VJT 30** CMD5-CMD6 98,9 KX231824.1 VJT 8 Aspergillus tubingensis ITS1-ITS2 100 KY612372.1 VJT 16 ITS1-ITS4 100 MK166185.1 **PSJ 16** 98,9 MK166185.1 βt2a-βt2b **PSJ 12** ITS1-ITS4 100 MN634560.1 Arthrinium gutiae PSJ 143 ITS1-ITS4 100 MN634560.2 **PSJ 13** Byssochlamys spectabilis 99 ITS1-ITS4 MW335157.1 PSJ 140 Canariomyces subthermophilus ITS1-ITS4 98 MK926804.1 100 PSJ 135 Chaetomium cochliodes ITS1-ITS4 MH590621.1 **PSJ 40** ITS1-ITS4 99 MH550490.1 KU571511.1 PSJ 40-2 Chaetomium acropullum ITS1-ITS4 99 **PSJ 83** ITS1-ITS4 99 KU571511.1 ITS1-ITS4 100 **PSJ 119** Corynascus verrucosus KY065360.1 ITS1-ITS4 98 GU192459.1 **PSJ 15** 99.9 PSJ 15-1 Chrysonilia sitophila ITS1-ITS4 GU192459.1 99,43 VJT-4 Corynascus sepedonium ITS1-ITS4 MK919294.1 VJT-3 ITS1-ITS4 99,3 KY039309.1 Cladosporium cladosporioides

**Table S1**. Identification of the fungi isolates form dry tomatoes, determined by amplifying Internal transcribed spacer 1 (ITS1) and ITS2 regions and the 5.8S ribosomal DNA (rDNA) region,  $\beta$ -tubulin and Calmodulin gene and nucleotide sequences.

PSJ 55		ITS1-ITS4	99	MF359643.1
PSJ 96	Eutypella microtheca	ITS1-ITS4	100	MH864886.1
PSJ 98		ITS1-ITS4	99	MH864886.
PSJ 151	Gymnascella dankaliensis	ITS1-ITS4	100	AY304514.1
PSJ 86		ITS1-ITS4	99	MN418435.
PSJ 70	Penicillium citrinum	ITS1-ITS4	99	MG575517.
PSJ 73		ITS1-ITS4	100	LC514694.1
PSJ 76	Ovatospora medusarum	ITS1-ITS4	100	MH860651.
PSJ 110	Psathyrella candolleana	ITS1-ITS4	99	MT424873.
VJT-22		ITS1-ITS4	99,7	LC514326.1
VJT-25	Rhizopus oryzae	ITS1-ITS4	99,7	MT603963.
VJT 11		ITS1-ITS4	99,6	LC514321.1
PSJ 87-1	Stemphylium eturmiunum	ITS1-ITS4	100	MW883450.
PSJ 3		ITS1-ITS4	100	MG065802.
PSJ 51		ITS1-ITS4	100	MG065802.
PSJ 102	Stemphylium vesicarium	ITS1-ITS4	100	MW245000.
PSJ 81		ITS1-ITS4	100	MN328401.
PSJ 58	Trametes elegans	ITS1-ITS4	100	MT597442.
PSJ 137	Trimmatothelopsis sp.	ITS1-ITS4	95	MK948457.