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ORIGINAL ARTICLE

Low-temperature, low-pressure gas plasma application on *Aspergillus brasiliensis, Escherichia coli* and pistachios

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Abstract

Aim: The aim of this study was to investigate the effect of plasma-enhanced chemical vapour deposition (PECVD) treatment on selected bacteria and spores and to contribute to the understanding of the synergistic effect of UV-directed plasma.

Methods and Results: The experiments were conducted on pure cultures of *Aspergillus brasiliensis* and *Escherichia coli* and on naturally contaminated pistachios that were exposed to pure oxygen-, pure argon- and to a mixture of oxygen–argon-generated plasma for different treatment times and at different micro-organism concentrations. Optical emission spectroscopy (OES) measurements were performed to observe the active species in the plasma. After exposure, the effectiveness of decontamination was assessed through microbiological techniques by calculating the growth reduction on a logarithmic scale. A treatment time of 30 min resulted in a 3·5 log reduction of *A. brasiliensis* using pure oxygen or argon, while treatment times of 5 min, 1 min and 15 s resulted in a 5·4 log reduction using a mixture of argon and oxygen (10 : 1 v/v). Treatment times of 1 min and 30 s resulted in a 4 log reduction of *E. coli* with oxygen and argon, respectively, which led to a complete elimination of the micro-organisms. Two-log reductions of fungi were achieved for pistachios after a treatment time of 1 min.

Conclusions: These results suggest that this newly designed plasma reactor offers good potential applications for the reduction in micro-organisms on heat-sensitive materials, such as foods. The plasma that was generated with Ar/O_2 was more effective than that which was generated with pure oxygen and pure argon.

Significance and Impact of the Study: An improvement in the knowledge about PECVD mechanisms was acquired from the chemical and biological points of view, and the suitability of the method for treating dry food surfaces was demonstrated.

Introduction

For thousands of years, techniques such as drying, salting and smoking have been used for food preservation and to prolong food shelf life and safety. Food safety is a critical issue for consumers and the food industry because microbiological contamination of food causes a considerable social and economic burden on health care (Yun *et al.* 2010). In Europe, 27 member states have reported cases of food-borne diseases that include 5648 food-borne outbreaks that affected 69 553 people and resulted in 7125 hospitalizations and 93 deaths in 2011 (European

Food Safety Authority (EFSA) 2013). Food-borne diseases are a major public health problem in Europe, and the World Health Organization (WHO) concluded that the incidence of food-borne diseases is a growing public health problem in developed and developing countries (World Health Organization 2008). In view of the increasingly massive requests for fresh, ready-to-eat products due to lifestyle changes and the limitations of the current storage systems, recent studies have suggested that sterilization with low-pressure cold plasma could be a viable alternative to the traditional methods for the decontamination of heat-sensitive materials or food because this technique has been proven to be capable of eliminating bacteria on surfaces without altering the substrate (Niemira 2012; Bermúdez-Aguirre *et al.* 2013).

Critical analysis of the literature testifies to the effectiveness of this technology and its applicability in various industrial sectors (e.g. microelectronics, textiles, decoration, paper, cultural goods, pharmaceutical packaging and industrial packaging), including food (Fridman 2008; Ziuzina *et al.* 2012; Mirjalili and Karimi 2013).

Plasma belongs to a class of 'nonthermal' disinfection techniques called advanced oxidation processes (AOPs), which are considered 'clean technologies' (Phull *et al.* 1996; Bhatkhande *et al.* 2003; Singh *et al.* 2003; Rodriguez-Romo and Yousef 2005; Moreau *et al.* 2008; Rodriguez-Romo and Yousef 2010).

Because of its compositions (photons, ions, electrons, radicals, UV, free radicals and ROS), plasma sterilization works differently and more accurately than other methods. Micro-organisms are bombarded by plasma-produced free radicals that cause irreparable injury to cell membranes, which results in the rapid destruction of the cells. Under the appropriate conditions, this technique can work at relatively low temperatures (<50°C); therefore, it is possible to achieve optimal results with a fine selection of operative parameters (RF power, gas flow, pressure and electrode distance) (Moisan *et al.* 2001; Kim and Kim 2006; Moreau *et al.* 2008).

Numerous scientific studies have demonstrated the capacity of plasma to destroy even highly resistant micro-organisms, such as spore-forming bacteria (*Clostridium perfringens*, *Bacillus cereus*), fungi, *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes, Vibrio parahaemolyticus* and especially entero-haemorrhagic *Escherichia coli,* which is responsible for more than 90% of food-poisoning cases and can survive for long periods under adverse conditions and refrigeration temperatures (Purevdorj *et al.* 2003; Schneider *et al.* 2005; Deilmann *et al.* 2008; Roth *et al.* 2010a,b; Mols *et al.* 2013).

The mechanisms by which plasma interacts with biological materials (prokaryotic and eukaryotic cells) and inactivates them are currently under investigation. It is well known that nonthermal plasmas can act as effective media for sterilization due to the germicidal action of several plasma components, including the physical effects of ultraviolet radiation and charged particles, such as ions and electrons, and chemical effects, such as the production of chemically active radicals.

Moisan *et al.* (2001) proposed a model for sterilization with low-pressure plasma that was derived from the observation of two sequential phases of rapid and slow inactivation of micro-organisms and from the properties of plasmas. The corresponding inactivation rates are limited by the action of UV radiation for fast inactivation and reactive particle-mediated erosion of biological material for slow inactivation. Mogul *et al.* (2003) showed that low-pressure oxygen plasma could degrade lipids, proteins and DNA of *Deinococcus radiodurans*. Kim and Kim (2006) reported the inactivation of enzyme activity and accumulation of ROS in *E. coli* after treatment with helium–oxygen atmospheric-pressure plasma. Exposure of *E. coli* cells and the spores of *Bacillus globigii* to atmospheric-pressure plasmas caused mutations in surviving cells, which led to metabolic changes (Laroussi *et al.* 2002, 2006; Bermúdez-Aguirre *et al.* 2013). Nevertheless, the details of the interactions of plasma agents with the different components of bacterial cells or spores are unknown. In particular, questions arise regarding which components of a cell or spore are the primary targets and which agents are the most effective in the inactivation process (Roth *et al.* 2010a).

Two studies (Basaran *et al.* 2008; Selcuk *et al.* 2008) reported the application of low-pressure cold plasma on grain, legumes and nuts that were infected with *Aspergillus* spp., *Aspergillus parasiticus* and *Penicillium* spp. The authors achieved a 1–5 log reduction in contamination after a treatment time of 5–20 min with air gas plasma and SF_6 plasma. The reduction of surface fungal contamination depended on the seed surface, plasma gas type, plasma treatment time and micro-organism concentration; thus, this technique needs further investigation for its application to food.

The aim of this study was to test the effectiveness of low-pressure cold plasma (LPCP) using various gases on the inactivation of fungi and bacteria and to contribute to the comprehension of the synergistic effect of UV and directed plasma. The experiments were initially conducted on pure cultures of *Aspergillus brasiliensis* and *E. coli* to investigate the relationship between the degree of inactivation and the plasma operating conditions; the experiments were then conducted on naturally contaminated pistachios to assess the feasibility of using this technology in the food industry to offer consumers a safer product.

Materials and methods

Low-pressure cold plasma (LPCP) device

A PECVD IV320 type twin sputtering chamber of our own design (Fig. 1) was employed to perform the experimental tests.

The PECVD chamber contains a circular water-cooled sample holder (150 mm in diameter) with a 200 mm diameter, circular gas shower as a counter electrode; this device is powered by a 600 W, 15·56 MHz RF power generator. Below, the sample holder is wired to a 750 W ± 600 V DC generator (which can be shortened if mass connection is needed). The distance between the two electrodes can be easily adjusted but was kept fixed at 20 mm for the experiments to achieve good plasma glow region immersion.

The PECVD chamber vacuum system consists of a first pumping line containing a 230 l s^{-1} turbo pump, followed by a standard rotary vane pump and a parallel second line with a rotary vane pump that is directly connected to a vacuum chamber through an automatic on–off valve. The vacuum chamber can be isolated from the first pumping line with a manually operated gate valve. Because of the extreme release of gas adsorbed on organic samples (outgassing), the needle valve was removed to reduce pumping resistance and to increase the flow rate.

Finally, the last modification involved a pressure threshold value for the switch on the turbo pump, which was increased to start the turbo pump earlier and to reduce the pumping time.

A generic process begins by pumping the PECVD chamber down to a pressure of $<5 \times 10^{-4}$ mbar through the first line to evacuate the atmospheric air. When low pressure is achieved, the second line is switched on, and the gate valve is closed. The pressure is then increased with the inflow of the process gas up to the final pressure of the target process. After pressure stabilization, the RF power is turned on at the desired power level, and at plasma ignition, the sterilization process begins. When the treatment time ends, the RF power is turned off, the second pumping line is closed and the gate valve is opened. The first vacuum line pumps down to below 5×10^{-4} mbar of the process gas, and finally, the atmospheric pressure is restored for the chamber opening operation. The process temperature is maintained near environmental levels (25°C).

Discharge operating conditions include the flow rate of the gas, power and exposure time. The trials were carried out with pure argon, pure oxygen and a mixture of argon and oxygen (1 : 1, 2 : 1 and 10 : 1 v/v) as the process gases. Regarding the operational limits and safety conditions of the PECVD plasma process, the first process started with 80 W of RF power, an electrode distance of 30 mm and a working pressure of $1{\cdot}3 \times 10^{-1}$ mbar. From the second process onward, the RF power was increased to 100 W, the electrode distance was set to 20 mm and the pressure was stabilized to $3{\cdot}0 \times 10^{-1}$ mbar. This procedure allowed for better configuration and stability of the glow discharge.

To increase ion bombardment on the sample, some treatments introduced a bias voltage of −50 or −100 V

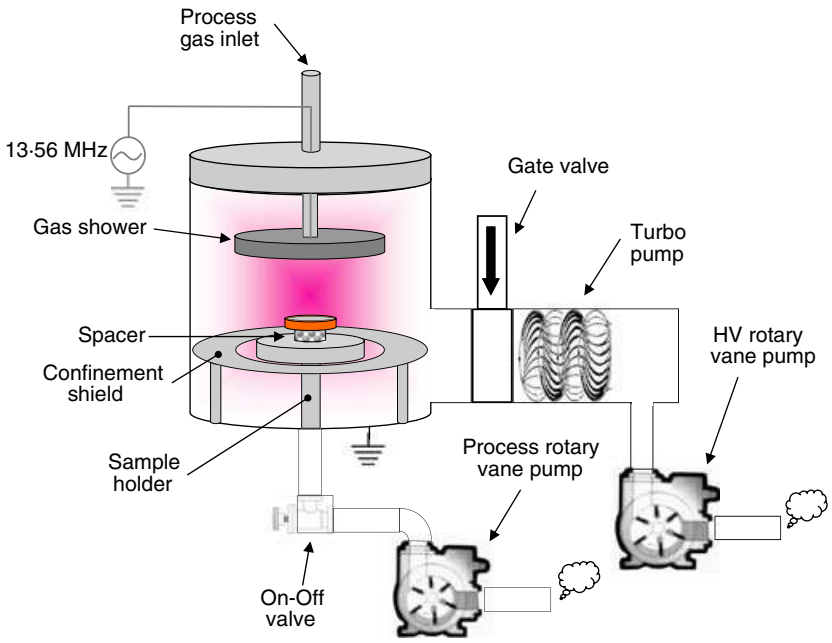

Figure 1 Schematic of the PECVD that was used during the tests.

for the sample stage with respect to the chamber reference (0 V). During these experiments, the process temperature was 40°C. Studies of the type of radical generated by the plasma have been carried out by optical emission spectroscopy (OES) to investigate the features of glow discharge. The measurements were performed with an OCEAN Optic spectrometer LIBS2500 2plus-optic probe QP600-2-SR/BX using an integration time (optical scan) of 100 ms.

Sample preparation

For the experiments described in Table 1, pure cultures of *A. brasiliensis* and *E. coli* were used. *A. brasiliensis* spores were lyophilized at $4{\cdot}7 \times 10^6$ CFU pellet^{-1} (certificate—ATCC 16404, lot 392141, MicroBioLogics USA, exp 03/2011), and the *E. coli* strain was lyophilized at $5{\cdot}5 \times 10^7$ CFU pellet^{-1} (certificate—ATCC 8739, lot 483361, MicroBioLogics USA, exp 09/2010).

Aspergillus brasiliensis and *E. coli* pellets were reactivated in 100 ml of sterile Ringer's solution (LAB M, lot 099882, exp 10/2010) for at least 30 min, as indicated by the manufacturer. Aliquots of the cultures were diluted in sterile Ringer's solution to achieve final cell concentrations of approximately 10^5 CFU ml^{-1} (range *A. brasilensis*: 4·23–5·46 log CFU ml^{-1}; *E. coli*: 4·26 log CFU ml^{-1}) and 10^3 CFU ml^{-1} (range *A. brasiliensis*: 3·55–3·60 log CFU ml^{-1}).

For each experiment, 100 ml of 10^5 or 10^3 CFU ml^{-1} of *A. brasiliensis* or *E. coli* suspensions was filtered on cellulose acetate filters (0·45 μm nominal porosity, 47 mm diameter; Sartorius AG, Lot 0607, exp 06/2011). The filters were then placed in sterile Petri dishes and dried under a laminar flow hood for at least 30 min before the plasma treatment. Exposure times of the plasma treatments to the membrane samples were 1, 5, 15 and 30 min for *A. brasiliensis* and 15, 30, 60 and 180 s for *E. coli*. Unexposed membrane samples were the control used in each test. They were utilized for the evaluation of the initial concentration of *A. brasiliensis* and *E. coli*.

For the evaluation of the effectiveness of the plasma process on raw, peeled, roasted and salt-free naturally contaminated pistachios, five experiments were performed, as shown in Table 2. Aliquots of 10 g of pistachios were put in sterile Petri dishes until the treatment. The plasma treatment exposure times were 1, 3 and 15 min, and the pistachios were not turned during the treatment because they cannot be turned during an industrial process. Unexposed pistachios (10 g) were utilized for the evaluation of the initial concentration of fungi (control). Pure oxygen and a mixture of argon and oxygen were used as the plasma gas without bias voltage. Each experiment on pure cultures and pistachios was conducted in triplicate.

Sample analysis

Plasma-exposed filters and unexposed filters (controls) were transferred to sterile Falcon tubes containing 10 ml of sterile Ringer's solution and were then placed in an ultrasonic sonicator for 5 min to allow for the detachment of *A. brasiliensis* and *E. coli* from the filters and to bring them into solution.

Aliquots of *A. brasiliensis* cell suspensions (300 μl dilutions for each treatment time) were spread on Sabouraud Dextrose Agar CAF 500 (Biolife, 8P5302 lot, exp 12/2011) plates in three replicates. The plates were then incubated

Table 1 Description of the experiments conducted on *Aspergillus brasiliensis* and *Escherichia coli*

Experiments	Micro-organism (CFU)	Gas flow (sccm)	Pressure (mbar)	Electrode distance (mm)	Temperature	RF power (13·56 MHz)
Test 1	*A. brasiliensis* (10^5)	O_2 (2·5)	$1{\cdot}3 \times 10^{-1}$	30	25°C	80 W
Test 2	*A. brasiliensis* (10^5)	O_2 (2·8)	3×10^{-1}	20	25°C	100 W
Test 3	*A. brasiliensis* (10^5)	O_2 (2·8)	3×10^{-1}	20	25°C	100 W Bias voltage –50 V
Test 4a/4b	*A. brasiliensis* (10^5 and 10^3) *E. coli* (10^5)	O_2 (3·0)	3×10^{-1}	20	25°C	40 W Bias voltage –100 V
Test 5a/5b	*A. brasiliensis* (10^5 and 10^3) *E. coli* (10^5)	Ar (3·0)	3×10^{-1}	20	25°C	40 W Bias voltage –100 V
Test 6	*A. brasiliensis* (10^5)	O_2 (2·3) Ar (30)	2·5	20	25°C	150 W
Test 7	*A. brasiliensis* (10^5)	O_2 (2·3) Ar (30)	2·5	20	25°C	300 W
Test 8	*A. brasiliensis* (10^5)	O_2 (2·3) Ar (30)	2·5	20	25°C	400 W

Table 2 Description of the experiments conducted on pistachios

Experiments	Gas flow (sccm)	Pressure (mbar)	Electrode distance (mm)	Temperature	RF power (13·56 MHz)	Exposure time (min)
Test 1 P	O_2 (20)	3×10^{-1}	20	25°C	100 W	15
Test 2 P	O_2 (10)	3×10^{-1}	20	25°C	100 W	15
Test 3 P	O_2 (2·3) Ar (3·7)	2·5	20	25°C	100 W	15
Test 4 P	O_2 (1·7) Ar (3·9)	2·5	20	25°C	300 W	3
Test 5 P	O_2 (2·3) Ar (30)	2·5	20	25°C	300 W	1

at 25°C for 5 days to allow for the development of mycelia and a correct count of CFUs.

Aliquots of *E. coli* cell suspensions (1 ml dilutions for each treatment time) were plated in duplicate on Tryptone Soy Agar plates (Lab M, 05778/309 lot, exp 09/2013) and incubated for 48 h at 37°C.

Treated and untreated (control) pistachios were transferred to sterile Stomacher bags with 90 ml of sterile Ringer's solution and were extracted for 3 min. Aliquots of pistachio cell suspensions (300 μl and 1 ml of dilutions for each treatment time) were spread in three replicates each on Sabouraud Dextrose Agar CAF 500 plate (SDA) for fungal counts. SDA plates were incubated at 25°C for 5 days.

Statistical analysis

The statistical analyses were performed using the statistical package SPSS 17·0 (SPSS for Windows, Chicago, IL) and Spearman's test.

Results

Aspergillus brasiliensis

The results obtained from the individual tests with low-pressure plasma are reported in Table 3. The first experiments were performed using a pure strain of *A. brasiliensis* at a concentration of 10^6 CFU pellet^{-1} that was revitalized in Ringer's solution and filtered through a cellulose acetate filter with a porosity of 0·45 μm. During the first test (test 1), decreases in concentration of 1·54, 1·63 and 1·48 log were obtained for *A. brasiliensis* at 5, 15 and 30 min, respectively, and these results appeared to not be significantly dependent on the exposure time.

To increase the effectiveness of the treatment, during the second test (test 2), the process parameters of the machine were changed.

The pressure was increased to $3{\cdot}0 \times 10^{-1}$ mbar (approximately 225 mTorr), and the power was changed to 100 W. In test 2, reductions in the concentration of *A. brasiliensis* of 1·82 log after 5 min of exposure, 2·88 log after 15 min and 3·45 log after 30 min were obtained (Table 3).

To increase ion bombardment on the sample, test 3 was performed by introducing a bias voltage of −50 V at the sample stage with respect to the chamber reference (0 V).

A 3·23 log reduction of *A. brasiliensis* was achieved after 30 min of treatment, but a negligible reduction occurred after 15 min of exposure. Another experiment (test 4a/4b) was performed to improve the supplementary ion bombardment by introducing a bias voltage of −100 V and to assess whether the initial concentration of the spores on the filter had an inhibitory effect on the efficacy of plasma sterilization. Assuming that a high concentration of spores on the filter could impede the access of the plasma to the lower layers (Leipold *et al.* 2010), two filters, in which solutions with concentrations of 10^5 and 10^3 CFU ml^{-1} had been filtered, were exposed to the plasma treatment. As shown in Table 3, test 4a/4b demonstrated that treatment with plasma was much more effective for the filters on which the concentration of spores was 10^3 CFU ml^{-1}. Specifically, after 30 min of treatment, a 1·83 log reduction was observed for the filters with initial spore concentrations of 4·81 log CFU ml^{-1} *vs.* a 3·60 log reduction for the filters with initial spore concentrations of 3·60 log CFU ml^{-1}. A subsequent test (test 5a/5b, Table 3) on *A. brasiliensis* was conducted to determine whether the type of gas used for generating plasma could influence the sterilization efficiency of the treatment. To test this hypothesis, plasma was generated with pure argon, and filters with two different spore concentrations (10^5 and 10^3 CFU ml^{-1}) were treated. Test 5a/5b showed that even in this case, the treatment with plasma was more effective for the filters on which the concentration of spores was lower (Table 3). After 30 min of treatment, a 2·93 log reduction occurred for the filters with initial spore concentrations of 5·32 log CFU ml^{-1} *vs.* a 3·55 log reduction for the filters with initial spore concentrations of 3·55 log CFU ml^{-1}; the latter outcome was deemed a total

Table 3 Results of the experiments on *Aspergillus brasiliensis* and mean reduction (SD in brackets)

Exposure time	Test 1	Test 2	Test 3	Test 4a	Test 4b (10^3)	Test 5a	Test 5b (10^3)	Test 6	Test 7	Test 8
Control	4·23 (0·23)	5·70 (0·29)	4·94 (0·26)	4·81 (0·21)	3·60 (0·19)	5·32 (0·30)	3·55 (0·23)	5·46 (0·29)	5·44 (0·32)	5·44 (0·30)
Reduction (log CFU ml^{-1})										
15 s	NT*	NT*	NT*	NT*	NT*	NT*	NT*	NT*	NT*	5·44 (0·29)
1 min	1·15 (0·08)	0·74 (0·04)	0·24 (0·01)	0·59 (0·07)	1·83 (0·13)	1·77 (0·07)	1·59 (0·09)	NT*	5·44 (0·33)	NT*
5 min	1·54 (0·10)	1·82 (0·16)	0·48 (0·03)	0·62 (0·06)	1·54 (0·12)	1·63 (0·10)	2·23 (0·14)	5·46 (0·31)	NT*	NT*
15 min	1·63 (0·13)	2·88 (0·14)	0·86 (0·05)	1·66 (0·12)	2·44 (0·18)	2·10 (0·13)	2·14 (0·15)	NT*	NT*	NT*
30 min	1·48 (0·12)	3·45 (0·22)	3·23 (0·21)	1·83 (0·11)	3·60 (0·25)	2·93 (0·19)	3·55 (0·21)	NT*	NT*	NT*

*Not tested.

elimination of *A. brasiliensis* that was detectable with the microbiological method used.

The Spearman's correlation was calculated between the fungal concentrations and exposure times to pure oxygen plasma, and it was found to be significant ($r = 0{\cdot}796$, $P < 0{\cdot}01$).

To characterize the slope of each segment, which we refer to as an *inactivation phase*, we used the time D that was required to decrease a given population of spores by a factor of 10 (90% reduction). The data suggested an inactivation kinetics consisting of three distinguishable, log-linear phases as shown in Fig. 2. A rapid inactivation phase ($D_1 = 1{\cdot}11$ min) at the beginning of plasma treatment, a second, slow phase ($D_2 = 17{\cdot}17$ min) and a third phase ($D_3 = 36{\cdot}60$ min). According to Moisan *et al.* (2001), temperature most likely plays an important role in the inactivation mechanism. In fact, when the substrate was at 40°C, the slopes of the survival curves were three, and the first phase showed a rapid inactivation. Both of these aspects suggest a thermally enhanced process. Tests 6, 7 and 8 were set up to increase the reduction of *A. brasiliensis* and to decrease the exposure time using a mixture of argon and oxygen (10 : 1 v/v) as the gas to generate plasma and increasing the RF power. These treatments were very effective in reducing the concentration of fungi on the filters, and a complete elimination of detectable *A. brasiliensis* was reached in 15 s by increasing the RF power to 400 W (Tables 1 and 3).

Escherichia coli

Other tests were performed using a pure strain of *E. coli* at a concentration of 10^7 CFU pellet^{-1} that was revitalized in Ringer's solution and passed through a cellulose

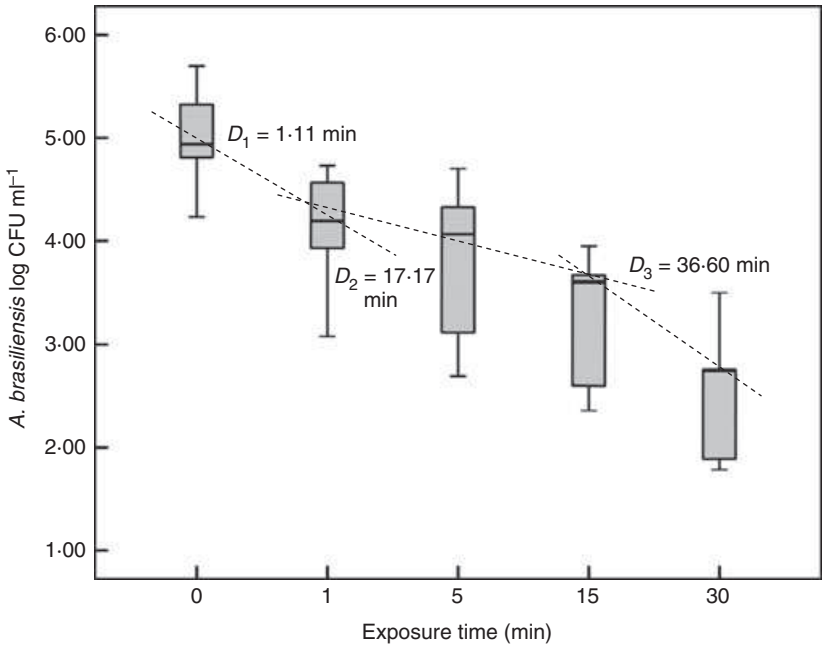

Figure 2 Box plot of *Aspergillus brasiliensis* concentrations at different exposure times in pure oxygen plasma at 40°C and inactivation kinetics (D_1, D_2, D_3).

acetate filter with a porosity of 0·45 μm. Because this organism is much more sensitive to treatment with plasma (Perni *et al.* 2007), it was exposed for a shorter time (15, 30, 60 and 180 s) than *A. brasiliensis*. Two tests (tests 4 and 5) were conducted under the same conditions as tests 4a and 5a on *A. brasiliensis,* by comparing argon and oxygen plasma sterilizations. The results are shown in Table 4 and highlight that both plasma treatments were very effective in reducing the concentrations of bacteria on filters. A 4·26 log reduction was achieved after 60 s of treatment with oxygen plasma and after only 30 s of exposure to argon plasma; this outcome represented the complete elimination of detectable *E. coli* in both treatments. In the test 5, only 3·96 log reduction was achieved after 60 s of treatment with argon plasma, but it was probably due to the biological variability of the pure culture used. The Spearman's correlation was calculated between the bacterial concentrations and exposure times to pure oxygen plasma, and it was found to be significant ($r = 0{\cdot}876$, $P < 0{\cdot}01$). As shown in Fig. 3, the inactivation kinetics consisted of three log-linear phases ($D_1 = 5{\cdot}21$ s; $D_2 = 51{\cdot}25$ s; $D_3 = 289{\cdot}08$ s) according to Moisan *et al.* (2001). Additionally, in this case, the first phase showed a rapid inactivation, and the third phase increased, suggesting a thermally enhanced process.

Pistachios

The food tests used pistachios that were naturally contaminated with fungi (range: 5·67–7·96 log CFU g^{-1}) and micro-organisms (range total plate count: 5·10–9·28 log CFU g^{-1}). In light of the results that were obtained on pure cultures, the pistachios were subjected to plasma generated under the same test conditions as test 2 and test 7. To evaluate the effectiveness of plasma in reducing the concentrations of fungi present on the pistachios, plates containing 10 g of contaminated pistachios were subjected to treatment with low-pressure oxygen plasma for 15 min as reported by other authors (Basaran *et al.* 2008; Selcuk *et al.* 2008) and then with low-pressure argon/oxygen (1 : 1, 2 : 1 and 10 : 1 v/v) plasma for 15, 3 and 1 min. The results are shown in Table 5. During the tests, visible changes in the treated *vs.* untreated pistachios were not apparent. The results obtained during the tests that were conducted with pure oxygen plasma (1P – 2P) showed a low reduction in the fungal population that was present on the pistachios, while the reduction in the fungal population reached 2 log (5P) on the pistachios that were treated for 1 min with argon/oxygen (10 : 1 v/v) plasma.

Table 4 Results of the experiments on *Escherichia coli* and mean reduction (SD in brackets)

Exposure time (seconds)	Test 4	Test 5
Control	4·26 (0·26)	4·26 (0·29)
Reduction (log CFU ml^{-1})		
15	1·97 (0·09)	3·79 (0·24)
30	2·40 (0·15)	4·26 (0·29)
60	4·26 (0·27)	3·96 (0·18)
180	4·26 (0·30)	4·26 (0·21)

Optical emission spectroscopy (OES)

To investigate the degree of ionization and dissociation with the glow discharge, OES was used, and the intensities of the molecular, atomic and ionic species that were observed in the plasma were measured. Figure 4 shows the optical spectra of the O_2, Ar and Ar/O_2 mixtures (10 : 1 v/v).

To reduce air contamination, the chamber was evacuated twice and subsequently filled with the process gas. However, impurities were inevitably present when we used the Ar and O_2 mixture. In fact, in this case, the optical emission spectra revealed traces of N_2 (second positive system). The results are shown in Fig. 4a, b, c. Figure 4a shows an optical emission spectrum measured from 200 to 1100 nm for pure O_2 (2·8 sccm) at 100 W of input power and $2{\cdot}9 \times 10^{-1}$ mbar. As shown in the figure, in the pure O_2 discharge, peaks representing the NO γ system (247 nm, very low), O^{2+} (524, 631 nm), OH Angstrom system (306 nm) and O* (394, 437, 616, 777, 844 nm) were observed. Figure 4b shows an OES measured from 200 to 1100 nm for pure argon (3·0 sccm) at 40 W of input power and $3{\cdot}0 \times 10^{-1}$ mbar. In Fig. 4b, peaks for the CO Angstrom series (483, 520, 561 nm), CO^+ Comet-tail system (427 nm), C_2 Swan system (489, 516 nm), H Balmer series (α 656 nm; β 486 nm; γ 434 nm) and Ar 4p-4s, 5p-4s, 5d-4p, 4p-3d and 6s-4p transitions (420, 426, 696, 706, 727, 750, 763, 772, 794 and 811 nm) were observed. Figure 4c shows an OES measured from 200 to 1100 nm for a mixture of Ar/O_2 (30·0/2·3 sccm) at 300 W of input power 2·5 mbar. In Fig. 4c, peaks for the NO γ system (247 nm, very low), CO Angstrom series (483 nm), CO^+ Comet-tail system (427 nm), C_2 Swan system (489 nm), H Balmer series (α 656 nm; β 486 nm; γ 434 nm), Ar 4p-4s, 5p-4s, 5d-4p, 4p-3d, 6s-4p transition (420, 426, 696, 706, 727, 750, 763, 772, 794 and 811 nm), O* (777 and 844 nm) and traces of N_2 (second positive system) were observed.

Discussion

Several important mechanisms occur in the plasma sterilization process. These mechanisms include chemical

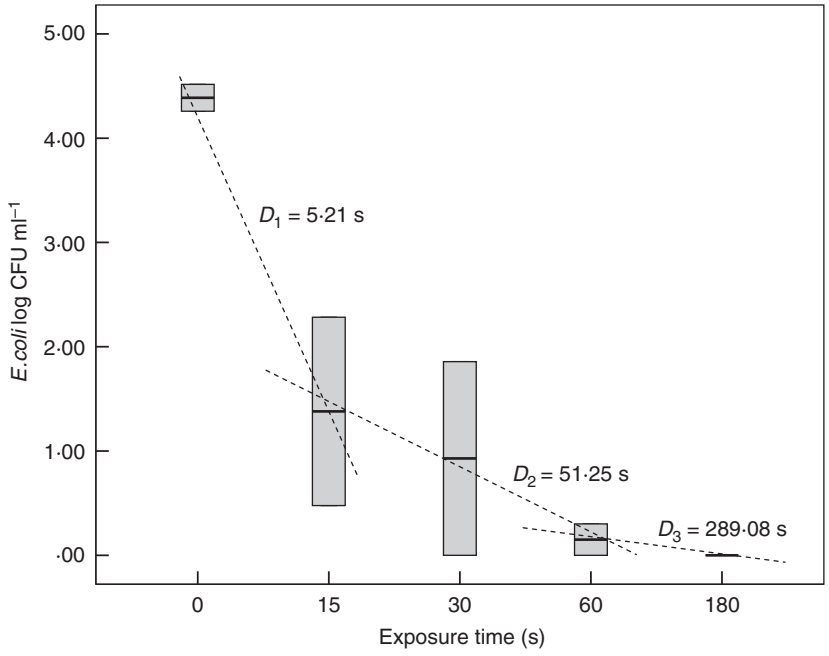

Figure 3 Box plot of *Escherichia coli* concentrations at different exposure times in pure oxygen plasma at 40°C and inactivation kinetics (D_1, D_2, D_3).

Table 5 Results of the experiments on pistachios and mean reduction (SD in brackets)

Experiments	Control (log CFU g^{-1})	Exposure time (min)	Reduction (log CFU g^{-1}) Fungi
Test 1 P	6·20 (0·45)	15	0·98 (0·21)
Test 2 P	7·84 (0·61)	15	0·63 (0·11)
Test 3 P	5·67 (0·38)	15	0·16 (0·04)
Test 4 P	7·96 (0·59)	3	1·16 (0·17)
Test 5 P	7·57 (0·52)	1	2·03 (0·31)

reactions with atomic oxygen, UV-induced damage or photodesorption and ion sputtering. The aim of this study was to contribute to the comprehension of the synergistic effect of UV and directed plasma. Three types of plasma gas were selected: the first was pure O_2 to explore the density of the metastable oxygen molecule and oxygen atom; the second was pure Ar (nondirectly oxidative plasma) to evaluate the role of metastable argon atoms and argon ions in the discharge condition and on the selected bacteria and spores and the last was an Ar/O_2 mixture (1 : 1, 2 : 1 and 10 : 1 v/v) to understand the role of a small percentage of oxygen in a mixture with argon.

The first test that was performed with pure oxygen as a gas produced evidenced that in our system, while operating at a pressure of $1{\cdot}3 \times 10^{-1}$ mbar, a power of 80 W and a distance of 30 mm (test 1), the glow discharge did not have good stability. The optimization of the power value (100 W), system pressure ($3{\cdot}0 \times 10^{-1}$ mbar) and distance (20 mm) achieved a decrease of 3·45 log after a 30 min exposure. To increase ion bombardment on the sample and, consequently, to promote surface chemical reactions between oxygen and lipopolysaccharides and peptidoglycans leading to the formation of volatile etch products, we used two treatments with bias voltages of −50 or −100 V for the sample stage with respect to the chamber reference (0 V).

The obtained results did not show the expected increase in efficiency, most likely because the ion flux did not interact in an optimal way with the sample surface or because the *A. brasiliensis* concentration per unit of surface was too high (several layers are formed), leading to poor diffusion of the treatment to the layers below as reported by Vleugels *et al.* (2005) in a study on the plasma inactivation of biofilm-forming bacteria.

To comprehend the cause, a test with 10^3 CFU ml^{-1} of fungi was performed that employed pure O_2 plasma and power at 40 W with a bias voltage of −50 V for the sample stage with respect to the chamber reference (0 V).

The obtained result was a decrease of 2·44 log after 15 min and 3·06 log (complete depletion) after 30 min.

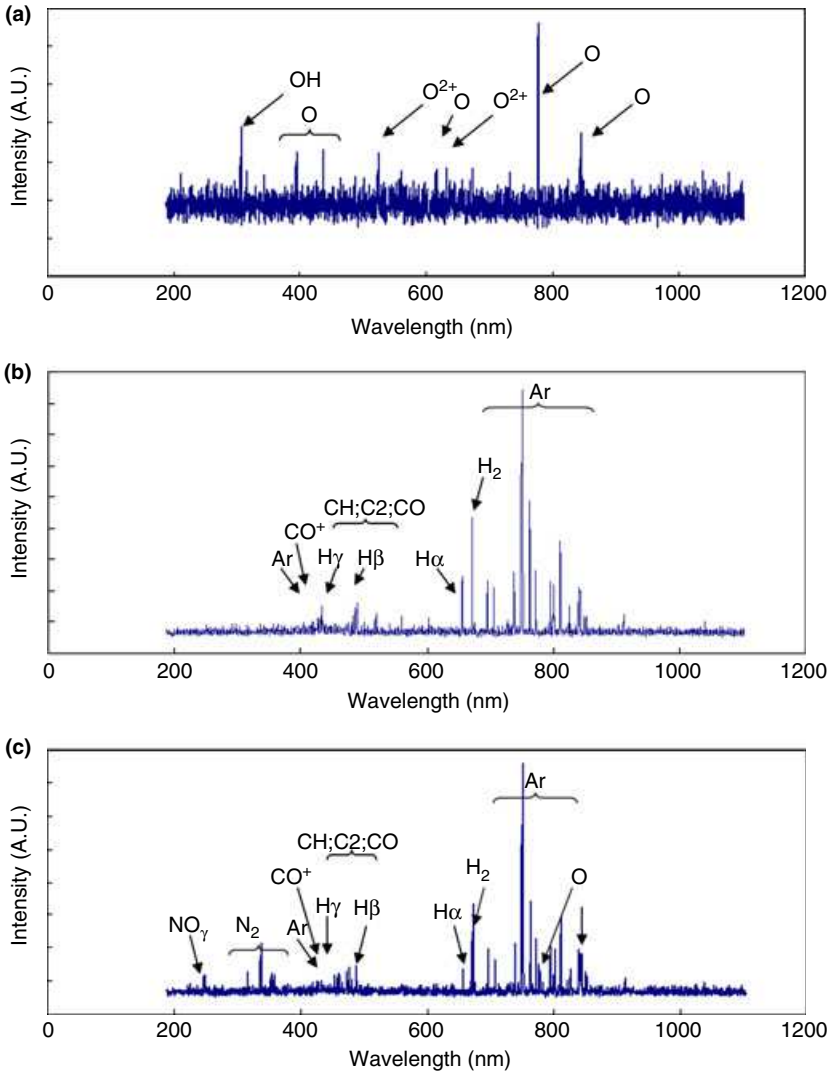

Figure 4 (4a) Optical emission spectrum of O_2 (2·8 sccm) at 100 W of input power and 0·29 mbar. (4b) Optical emission spectrum of Ar (3·0 sccm) at 40 W of input power and 0·30 mbar. (4c) Optical emission spectrum of Ar/O_2 mixture (30·0/2·3 sccm) at 300 W of input power and 2·50 mbar.

To investigate the role of UV, we compared the sterilization efficacy of pure oxygen with that of pure argon in the discharge.

We performed two experimental tests (5a and 5b) that employed pure argon, with power at 40 W and bias voltage of −100 V for the sample stage with respect to the chamber reference (0 V). In test 5a, the contamination was 5·32 log CFU ml^{-1}, while in 5b, this value was 3·55 log CFU ml^{-1}.

Similar to the results of Moreau *et al.* (2008), our results of test 5a showed partial killing from the pure argon plasma by the action of UV photons. Nevertheless, OES spectroscopy revealed organic material traces in the plasma phase due to the layer-by-layer erosion of living organisms by ions.

On the contrary, test 5b achieved a decrease of 3·55 log after 30 min and more than 2 log after 15 and 5 min of treatment. Pure oxygen plasmas led to three segment plots that had a large number of survivors remaining with three distinguishable log-linear phases (according to Moisan *et al.* 2001) that were most likely due to the temperature of the substrates (+40°C). Furthermore, the second phase could be related to spores/bacteria that were shielded by others and required longer irradiation time to accumulate a lethal UV dose.

The last three tests (6, 7 and 8) were performed using feeding plasma with an Ar/O_2 (10 : 1 v/v) mixture and using increasing power (150, 300 and 400 W). The addition of O_2 led to the complete reduction of detectable *A. brasiliensis* in short exposure times (5 min, 1 min and 15 s). In this case, the role of the oxygen atoms is fundamental. The presence of a low concentration of O_2 in the Ar discharge with high UV emission intensity renders the synergistic effect between O atoms and UV photons efficient. Hence, adding O_2, which partially dissociates into O (as evidenced in OES spectra by peaks at 748, 777 and

845 nm), a synergistic effect occurs in which both UV photons and O atoms contribute to the inactivation of the fungi. Indeed, the amount of oxygen must be sufficient to have oxidizing power, while the argon must remain active in the plasma and have sufficient kinetic energy to stimulate the reactive oxygen species.

A general description of the possible mechanisms being triggered on the surface of living organisms was described by Moreau *et al.* (2008) and von Keudell *et al.* (2010). They suggest that chemical sputtering is most efficient if argon with a small admixture of O_2 is used in the plasma discharge.

In our case, we also found a small percentage of residual N_2, argon ions and O, OH and NO radicals, as evidenced in Fig. 4. Thus, the efficient argon ion bombardment caused surface alteration of morphology and activation (by covalent bond breaking in the external cellular wall) of chemical bonds that reacted with O, OH and NO radicals and led to surface lesions by direct bombardment (etching, as revealed by the OES spectrum; see Fig. 4).

In addition, emissive de-excitation from the 4p manifold Ar (4p) $\rightarrow$ $Ar^m + h\nu$ (Gudmundsson and Thorsteinsson 2007) was the most important path for the creation of the metastable argon atom Ar^m (roughly 40% at 100 mTorr). This UV radiation is the main component of pure argon plasma and is one of the two exogenic causes of DNA damage by the Ar/O_2 mixture (Moisan *et al.* 2001; Niemira 2012).

With tests 6–8, we demonstrated that plasma generated with an Ar/O_2 (10 : 1 v/v) mixture is the most effective on *A. brasiliensis,* and a complete reduction was reached with the treatment for 15 s. This result could be due to the increase in power and the gas mixture that was used. Regarding *E. coli*, we reached complete elimination of detectable micro-organisms in 1 min with pure oxygen plasma and in 30 s with pure argon plasma. Similarly, effective treatment times were reported in Ziuzina *et al.* (2012). This group used a dielectric barrier discharge (DBD) device to generate air plasma for the treatment of a pure culture of *E. coli* in liquid media. They obtained the complete inactivation of bacteria (7 log CFU ml^{-1}) after 20–45 s of air plasma treatment.

Compared to the published literature, the abatement reached in the tests on pistachios was lower than the reduction that was obtained by Selcuk *et al.* (2008). They reported a 3 log reduction after 20 min of treatment using plasma generated with SF_6. The comparison with our results is not straightforward: they used different gas, equipment and process parameters; moreover, the experiment was performed on artificially contaminated beans and not on naturally contaminated pistachios. In Basaran *et al.* (2008), the authors treated peanuts, pistachios and hazelnuts that were artificially contaminated with *A. parasiticus* with plasma generated with air and SF_6 and achieved a 1·3 log reduction in 15 min and a 5 log reduction in 5 min. The same authors claimed to have more problems with the reduction in fungi on peanuts and pistachios because of the characteristics of the tegument of these two fruits. Specifically, the pistachio has a shell that is divided into two parts and does not protect from fungal attack. Additionally, the results obtained by Perni *et al.* (2008) demonstrated that the efficiency of sterilization with plasma treatment was greatly reduced when it was performed on the surfaces of fruits (mango and melon) rather than on nitrocellulose membranes. A more likely explanation for this phenomenon was that the microbial cells that were deposited onto the surface of the cut fruit tissue migrated away from the surface and into the interior of the tissue. This migration (internalization) would most likely take place along the interstitial spaces between the cells of the fruit.

We can conclude that this technology offers good prospects for use on different foods for human consumption, but it can only be applied to the food industry after other studies involving close collaborations between microbiologists, biochemists, physicists and engineers. This technique, when preceded by the appropriate studies to carefully select the parameters (i.e. gas type, pressure, frequency of electric field strength and temperature), could provide a sterilization process that is characterized by higher performance and economic and environmental sustainability. This result is dependent on the types of micro-organisms and substrates that are present and on balancing the effects of UV radiation, reactive species and free radicals.

Additional research will therefore uncover the ideal parameters by which to achieve the most effective killing of micro-organisms in foods to facilitate the use of this technology for the reduction of chemical contaminants, such as aflatoxin or pesticides, in food and to offer the consumer a safe product with the same organoleptic and nutritional characteristics. Finally, this technology should be applied to packaging materials to prolong shelf life, which would have a significant impact throughout the food industry sector and even greater benefits to the consumers, who may, in turn, find that available products have a prolonged shelf life, and for the distributors and the producers, who will likely experience smaller numbers of returned or eliminated products.

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Conflict of interest

No conflict of interest declared.

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