Atmosferic pressure non-thermal plasma: Preliminary investigation

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Abstract

Antibacterial activity of atmosferic pressure non-thermal plasma (APNTP) was assessed for bacterial, yeast and mold strains. This investigation is to be considered preliminary: a second step is envisaged in which the efficacy of the technique and the device will be assessed directly on food of animal and plant origin. The strains (ATCC or wild type) of *Listeria innocua*, *Escherichia coli*, *Salmonella thyphimurium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Proteus mirabilis* (bacteria); *Alternaria alternata*, *Aspergillus flavus*, *Cladosporium herbarum*, *Fusarium graminearum*, *Geotrichum candidum*, *Penicillium roqueforti*, *Rhizopus nigricans* (moulds); *Candida parapsilosis* and *Candida albicans* (yeasts) were subjected to plasma plume generated by the action of electric fields with a gas mixture (oxygen and helium) delivered for 5 min at a distance of 2 cm. Types of experiments were listed as following: microorganism at concentration 1×10^8 and 1×10^4 cfu on PCA (Plate Count Agar); *Listeria innocua* and *Salmonella thiphymurium* at concentration 1×10^4 cfu on semi-synthetic and synthetic medium; mycetes (moulds and yeasts) at concentration 1×10^8 and 1×10^4 cfu on SDA (Sabouraud Dextrose Agar).

The results obtained on the bacteria subjected to atmospheric cold plasma were evident on all the strains tested except for *Proteus mirabilis* (1×10^8 cfu), most evident at a concentration of 1×10^4 cfu, not only on culture media PCA but also on semi-synthetic medium and jelly meat-PCA medium. In spite of bacterial results, treatment with plasma plume did not decrease or inhibit of fungal growth. That means plasma plume was neither fungicidal nor fungistatic activities.

Introduction

Cold plasma was originally developed for ameliorating the printing and adhesion properties of polymers, increasing surface energy of materials and a variety of domains in electronics. It is commonly used to treat textiles, glasses, paper, and other products. A new research trend has suggested that cold plasma technology is a powerful and profitable technology for the food industry (Ekezie *et al.*, 2017).

Thermal processing remains the most widely used pasteurization and sterilization method for the inactivation of microorganisms and enzymes in foods. To overcome the concerns related to the poor quality of thermally processed foods such as nutrient losses and adverse effects on organoleptic quality, research in food science and technology over the last few decades has largely focused on the development of non-thermal technologies. Several new approaches, including high pressure processing (HPP), gamma irradiation, pulsed electric fields (PEF), ultraviolet light and sonication, have been successfully evaluated in a range of food products (Misra *et al.*, 2016).

However, some of these commercially available methods are limited in practice due to associated adverse perceptions (*e.g.* irradiation and electron beams), high initial investments required and incompatibility with in-line treatments (*e.g.* HPP), incompatibility for treatment of whole solid foods (*e.g.* PEF), lack of development in industrial scale equipment's for processing (*e.g.* ultrasound) and/or other constraints (Misra *et al*., 2016).

Recently, the field of cold plasma applications has rapidly expanded in many manufacturing industries, such as biomedical devices textiles, automotive, aerospace, electronics, and packaging materials (Bermudez-Aguirre, 2020; Govaert *et al*., 2020) furthermore into treatment of biological materials including foods to reduce microbial count to improve the shelf-life and safety of animal food (fish, meat, egg) and agricultural products (fresh vegetables) (Kim *et al*., 2020; Moutiq *et al*., 2020; Olatunde *et al.*, 2019; Georgescu *et al*., 2017; Pasquali *et al*. 2016; Trevisani *et al*., 2017; Mohamed *et al.*, 2021; Liao *et al*., 2017), degrade mycotoxin (Puligundla *et al*., 2020), inactivate enzymes (Kang *et al*., 2019), increase the concentration of bioactive compounds (Silveira *et al*., 2019), enhance antioxidant activity (Li *et al*., 2019), degraded pesticide residues (Phan *et al*., 2018) and food allergens (Venkataratnam *et al*., 2019) in food products.

The term "plasma" refers to a partially or wholly ionized gas composed essentially of photons, ions and free electrons as well as atoms in their fundamental or excited state possessing a net neutral charge. The plasma possesses a net neutral charge because the number of positive charge carriers is equal to the number of negative ones (Kudra *et al.*, 2009). Due to its unique properties, plasma is often referred to as the fourth state of matter according to a scheme expressing an increase in the energy level from solid to liquid to gas and ultimately to plasma. Two classes of plasma, namely thermal and non-thermal, can be distinguished based on conditions in which they are generated.

The use of sterilizing properties of plasma was first introduced at the end of the 1960's, patented in 1968 (Menashi, 1968) and the first study with plasma derived from oxygen was proposed in 1989: Nelson and Berger (Nelson *et al.*, 1989) showed that O_2 plasma could be a very efficient biocidal agent against bacteria. Thereafter, considerable research has been performed on the

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Key words: Atmospheric-pressure non-thermal plasma, Antimicrobial activity, Foodborne microorganisms.

Contributions: The authors contributed equally.

Conflict of interest: The authors declare no potential conflict of interest.

Funding: None.

Ethical approval and consent to participate: Written informed consent was obtained from the patient.

Availability of data and material: Data and materials are available by the authors.

Received for publication: 15 August 2021.
Accepted for publication: 1 September 2022.

Italian Journal of Food Safety 2022; 11:10043
doi:10.4081/ijfs.2022.10043

mechanisms of microbial inactivation by plasma agents. Cold plasma, indeed, showed potential for inactivation of a range of microorganisms, allergens and enzymes because it produces reactive species, such as reactive particles, free radicals, UV reactive species, and reactive elements of N_2, O_2, and H_2 (Hertwig *et al.*, 2018) that detrimentally interact with vital cellular biomacromolecules, such as DNA, lipids, proteins and enzymes in cell but without damage to nutritional and sensorial properties of food in the case of thermal treatment (Misra *et al.*, 2016).

The decontamination efficiency of non-thermal gas plasma treatments has been evaluated against gram-negative and gram-positive bacteria, spores, yeasts, molds and viruses (Montie *et al.*, 2000).

The ability to generate non-thermal plasma discharges at atmospheric pressure makes the decontamination process easier and less expensive.

Therefore, in this study, we assayed the biostatic and/or biocidal capacity of plasma on microorganisms of food interest.

Materials and Methods

The tested microorganisms included: gram-positive bacterial strains such as *Listeria innocua* (ATCC 33090), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212; gram-negative bacterial strains such as *Escherichia coli* (ATCC 35218), *Salmonella thyphimurium* (ATCC 13311), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 27853); seven wild-type mould strains such as *Alternaria alternata, Aspergillus flavus, Cladosporium herbarum, Fusarium graminearum, Geotrichum candidum, Penicillium roqueforti, Rhizopus nigricans,* and two wild-type yeast strains such as *Candida parapsilosis* and *Candida albicans*. All the strains were kept in microbanks at –20°C and before use were cultivated in BHI (Brain Heart Infusion) broth (for bacteria) and Malt broth (for mycetes) and incubated for 48 h at 37°C and 5 days at 25°C respectively. The final concentrations of 1×10^8 cfu/ml and 1×10^4 cfu/ml was measured with a spectrophotometer (at wavelength 600 nm).

The 1st test was carried out by aseptic streak the bacterial and fungal liquid cultures on PCA (Plate Count Agar) (bacteria) and SDA (Sabouraud Dextrose Agar) (fungi) media. After 5–7 min, plates were exposed aseptically to atmospheric plasma treatment generated by the action of electric fields on a gas mixture oxygen/helium (O_2/He) delivered for a time of 5 min at 2 cm from the source.

After incubation time, for bacteria 48 h at 30°C and for mycetes 5 days at 25°C, appeared colonies were recorded. All experiments were done at two different concentrations (1×10^4/ml and1×10^8/ml) in trireplicates.

For the 2nd and 3rd test, we proceeded as previously described for the preparation of the bacterial suspensions for two bacterial strains, *Listeria innocua* (ATCC 33090) and *Salmonella thiphymurium* (ATCC 13311) and two semi-synthetic media and one synthetic media were used. Semi-synthetic media were beef medium and chicken medium (Beef Medium included homogenized beef 160 g, meat extract 10 g, glucose 3 g, agar 10 g, distilled water 500 ml; Chicken Medium included chicken homogenized 160 g, meat extract 10 g, glucose 3 g, agar 10 g, distilled water 500 ml) and synthetic media were prepared by including in PCA the jellied meat (commercial purchase). After sterilization of media and aliquot in petri dishes, the bacterial suspensions at concentration 1×10^4/ml were smeared on the surface of the media. Then, they were exposed to the plasma plume under the same conditions as previous described and the petri dishes were incubated for 48 hours at 37°C. For all experiments, culture media without any treatment were used as control samples.

Results

The bacterial results showed that at less concentration of bacteria (1×10^4 cfu) the growth of all tested bacteria, regardless to gram-positive or gram-negative, was inhibited by plasma plume because there were considerable inhibition halos in compared with control samples. At high bacterial concentration (1×10^8 cfu) the reduction of growth of *Enterococcus faecalis*, *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhimurium* was not so marked and just some spots of inhibition halos observed. It is not surprisingly, because the concentration of bacteria were high and may have required either more time of exposure or high dosage of APNTP. In the case of *Listeria innocua* and *Salmonella typhimurium*, the results of the semi-synthetic culture media were satisfactory. Instead of it, on the PCA-jelly meat culture media the inhibition halos of growth were weak and barely perceptible.

The growth of all tested mycetes (fungi and yeasts, with both concentration 1×10^8 cfu and 1×10^4 cfu) showed no significant change in comparison to untreated samples as control, the treatment with the cold plasma plume was ineffective against the mycetes.

Discussion

Cold plasma is an emerging disinfection method that offers an exciting complementary or alternative non-thermal approach for reducing the microbial contamination of raw or fresh food products and packaging materials. There may be several other applications in relation to food systems, which remain unexplored. Various reactive species of plasma interact with the cells to cause permanent changes at cellular and molecular level, leading to inhibition.

The microorganisms tested showed different sensitivity to O_2/He plasma. For the bacteria, at the concentration of 1×10^8 cfu they were indifferent to plasma, except *Listeria innocua* and *Staphylococcus aureus*, but at the concentration of 1×10^4 cfu their growth was markedly arrested.

Gram-positive and gram-negative bacteria responded differently to plasma because their cell structures are different but evidently the efficacy of the plume overcame these differences, acting in both cases effectively. These findings supported the hypothesis that O_2/He plasma showed a sterilizing effect via oxygen radicals that can access and directly attack the cell wall. The interaction of ROS (Reactive Oxygen Species) with cell wall (peptidoglycan) of Gram-positive bacteria was investigated using atomic-scale simulations by Yusupov *et al.* (Yusupov *et al*., 2012, Yusupov *et al*., 2013): the study revealed the breakage of structurally important chemical bonds, including C-O, C-N and C-C.

The cell wall of fungi and yeasts has a different structure, consisting of chitin, glucans (beta-glucan) and mannans, offers a certain resistance to external injuries and is less prone to mechanical damages. This caused these microorganisms not to respond to the plasma plume.

Furthermore, through a series of studies, the inactivation of *Penicillium digitatum*, has been observed to be caused by atomic oxygen in low pressure plasma, while O_3 and NO have been found to play insignificant role (Hashizume *et al.*, 2013; Hashizume *et al.*, 2014; Iseki *et al*., 2011; Ishikawa *et al.*, 2014).

We did not obtain satisfactory results regarding the test with meat in jelly, and both *Listeria innocua* and *Salmonella typhimurium* developed on the culture medium: it is therefore possible that in this experimental condition the mixture of oxygen and helium was not effective or the time of administration of the plasma plume was

not sufficient or adequate. Nevertheless, this study is to be considered preliminary and needs further and careful appraisal. It should be kept in mind, however, that meat is considered a heat-sensitive food product and therefore ROS could potentially alter the function of biological membranes via interaction with lipids causing the formation of unsaturated fatty acid peroxides, oxidation of the amino acids in proteins (Misra *et al*., 2016) and development of oxidative rancidity (Varilla *et al.*, 2020).

Conclusions

In conclusion, APNTP using O_2/He can be an effective sterilizing agent against various food microorganism, without producing armful residues or exposing food materials to high temperatures or pressure, as in the case of conventional sterilization methods such as autoclaving, chemical treatment or high doses of irradiation (Laroque *et al*., 2022).

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