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Effects of atmospheric cold plasma on microbial growth of *Listeria innocua* and *Staphylococcus aureus* in ready-to-eat fish products

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Abstract

Food-borne pathogens, including *Staphylococcus aureus* and *Listeria monocytogenes*, in fish products account for death to a great extent. To increase shelf-life of foods and also preserve their sensory properties, plasma treatments have attracted more attention. The present study was intended to investigate the efficacy of atmospheric cold plasma (ACP) using argon and helium gas flows for ready-to-eat (RTE) fish products contaminated with *L. innocua* and *S. aureus* at short treatment durations of 4, 6 and 10 min. The results showed that argon plasma at each time point (4, 6, and 10 min) had a significantly inhibitory effect on the growth of *L. innocua*. Also, it reduced over a 10-minute treatment with helium plasma. There was a direct correlation between exposure time and antimicrobial efficiency of argon and helium ACP. The survival of *S. aureus* decreased by 0.19-1.04 Log cycles after argon plasma whereas helium plasma had a lower potential for growth reduction within the range of 0.03 and 0.55 Log cycles. It could be concluded that ACP is effective for lowering growth of different micro-organisms on RTE fish samples. *L. innouca* was considerably more sensitive than *S. aureus* to atmospheric pressure plasma (APP).

Keywords: Cold plasma, Argon, Helium, Microbial viability, Fish products

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Introduction

Food-borne pathogens in seafood, including fishes, have been recently considered as a critical factor of death, incidence of which has been reported sporadically in many regions across the globe by epidemiologists. Fast food and ready-to-eat (RTE) products are the most effective matrices spreading food-borne illnesses. Eating pathogens or their toxins in these products consequently led to infection and intoxication. Food processing environment usually acts as a means of pathogen transfer to the surface of food. *Staphylococcus aureus* and *Listeria monocytogenes* are two main pathogens that contaminate RTE seafood products due to unhygienic conditions. They are used as indicators of the suitable manufacturing process in food industries, particularly for RTE (Feldhusen, 2000; Rod *et al*., 2012; Zarei *et al*., 2012; Abdollahzadeh *et al*., 2016).

Physicochemical analyses have been indicated that seafood products are very prone to deterioration agents and need to be treated in order for shelf life to be extended (Özden and Erkan, 2010). If contamination occurs by pathogens and lipid oxidation, these products are more potent to cause off-odor and off-taste (Rostamzad *et al*., 2010). There have been diverse methods for preservation and decontamination in seafood industry. Preservative methods are divided into two categories including thermal and non-thermal treatments (Sofos and Geornaras, 2010). Of them, non-thermal methods not only extend shelf-life, but also protect food against deterioration without any adverse effects on sensory and nutritional properties. Ultrasound, high pressure technology, pulsed electrics field, pulsed light, cold plasma, and so forth are non-thermal methods widely used in food processing (Noriega *et al*., 2011).

Plasma is excited high energetic molecules, atoms, radicals and particles of gases, such as helium and argon, which emit infrared light in the range of visible spectra to ultra violet. This emerging technology can be put for a special use in industrial and trial scales. It has been employed for modern television, microcontrollers, and decontamination methods in hospitals, as well. There are two types of plasma treatment namely hot and cold plasma. In cold plasma, temperature of sample's surface is not raised higher than 50 °C (Ziuzina *et al*., 2013; Pankaj *et al*., 2014). Antimicrobial effects of this novel technology are owing to ions, radicals, and other compositions, which decontaminated surface of exposed food samples (Pankaj *et al*., 2014). Plasma treatments were typically conducted under vacuum conditions however an atmospheric pressure plasma (APP) system was lately established to diminish cost, enhance treatment speed, and expand its industrial applicability (Yoon and Ryu, 2007; Yun *et al*., 2010).

A great deal of literature has adopted cold plasma as an effective way to decontaminate surface of food products. Kim *et al*. (2011) utilized APP in an attempt to decontaminate surface of bacon samples; it was shown that viable counts of *E. coli, L. monocytogenese*,

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and *S. typhimuruim* were significantly affected following the treatment. Noriega *et al*. (2011) administered cold plasma in order for chicken skin and muscle to be disinfected from *L. innocua*; a tendency of growth inhibition by 3 logs was evident in microbial analyses. The aim of this study was to compare the effect of atmospheric cold plasma applying two different supply gases including argon and helium on the survival of *L. monocytogenes* and *S. aureus* inoculated on sea surface.

Materials and methods

Samples preparation and pathogens inoculation

Fish nuggets were purchased from local markets in Tehran, Iran, and then transported immediately to the laboratory in cooler boxes containing ice and stored under the refrigerator condition (4 °C) for subsequent use. The pure cultures of *S. aureus* ATCC 6538 and *L. innocua* ATCC 33090 (as a surrogate for *L. monocytogenes* (Leipold *et al*., 2010; Rod *et al*., 2012) were obtained from Iranian Research Organization for Science and Technology, Iran. Initially, *S. aureus* was grown in tryptic soy broth (TSB; Merck, Germany) at 37 °C for 18 h and subsequently underwent refrigerated centrifugation at 10,000 ×g for 10 min under aseptic conditions. *L. innocua* was cultivated in brain heart infusion Broth (BHI Broth; Merck, Germany) at 30 °C for 24 h. Fish nuggets were aseptically cut into sample sizes of 60×60 mm, which can be inserted into the cold plasma device. As for every strain, a total volume of 10 µl containing 10^9 CFU ml^{-1} was placed on 10 different areas on each sample. Bacterial strains were separately inoculated on different samples at the same time.

Treatment of atmospheric cold plasma

The custom-made cold plasma device included a ceramic tube with inner diameter of 1.5 mm for confining argon and helium flow and a concentric copper electrode (1 cm wide) that is connected to a high voltage power supply and wrapped up around the ceramic tube. Moreover, there was a disc electrode placed 2 cm downstream of the nozzle exit. The powered electrode was energized using a purpose-built high-voltage and AC power supply 12 and 22 kV for helium and argon, respectively. Viable excitation frequencies were 18 and 32 kHz for helium- and argon-based gas plasma, which were applied a short treatment duration of 4, 6 and 10 min. Helium (99.997% purity) and argon (99.997% purity) were fed through the hollow ceramic tube at the fixed flow rate of 6 L min^{-1}. The ionized gas emit from electrode united into the ambient air towards a point where the samples were placed (Ziuzina *et al*., 2013).

Microbial analysis

Following plasma treatment, a 5 g of the exposed samples was mixed with 45 mL of sterile saline by means of a mixer for two minutes. Thereafter, decimally diluted solutions were prepared using normal saline. A certain volume (0.1 ml) of the solution was

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spread out on tryptic soy agar (TSA; Merck, Germany) plates, which were further incubated at 37 °C for 48 h. Microbial counts were expressed as Log CFU g^{-1} (Lee *et al*., 2016).

Statistical analysis

Statistical analysis was carried out using SPSS 23.0 (SPSS Inc., Chicago, USA). Analysis of variance (ANOVA) and Turkey method were used at P-value below 0.05. Paired sample t-test was performed to examine the significant difference prior to and following plasma treatment. Any disparities between cell counts of Gram-negative and Gram-positive bacteria were tested by independent sample t-test.

Results

Growth reduction of *L. innocua* before and after the APP treatment is shown in Table 1. Argon plasma at each time point (4, 6, and 10 min) carried significantly negative effects on the growth of *L. innocua* (p<0.05). The initial population of *L. innocua* significantly (p<0.05) reduced by 0.19, 0.42, and 1.38 Log cycles following exposure to helium plasma for 4, 6, and 10 min, respectively (Table 1).

Table 1: The growth reduction of *Listeria innocua and Staphylococcus aureus* by atmospheric cold plasma treatment in seafood samples.

Plasma treatment	Exposure time (min)	*L. innocua* (Log cycle)	*S. aureus* (Log cycle)
Argon-based	4	0.77 ± 0.02^{c}	0.19 ± 0.01^{b}
	6	1.01 ± 0.01^{d}	0.32 ± 0.02^{d}
	10	2.18 ± 0.01^{f}	1.04 ± 0.00^{f}
Helium-based	4	0.19 ± 0.01^{a}	0.03 ± 0.02^{a}
	6	0.42 ± 0.00^{b}	0.16 ± 0.00^{c}
	10	1.38 ± 0.00^{e}	0.55 ± 0.00^{e}

Different letters within each column indicate statistically significant difference (p<0.05)

10-minute exposure to argon plasma caused the highest decrease in the microbial growth of *L. innocua* (p=0.001). It was found that plasma treatment with argon gas flow even at 4 min could effectively decrease *L. innocua* population up to 0.77 Log cycles (> 0.19 Log cycles by helium APP).

As for *S. aureus*, exposure to argon APP for 6 and 10 min was associated with a significant growth inhibition versus 4-min treatment (p<0.05). Cold plasma treatments with argon at 4 min displayed a non-considerable reduction around 0.19 Log cycles. With respect to helium plasma, only treatment for 10 min could yield a marked bacterial decrease (0.55 Log cycles) (p=0.004). Moreover, exposure time positively affected the growth inhibition. The microorganism survival decreased by 0.19-1.04 Log cycles after argon APP, whereas helium plasma showed a lower potential within the range of 0.03 and 0.55 Log cycles over a 10-min treatment period (Table 1). Dobeic *et al*. (2016) documented the beneficial effects of non-thermal plasma treatment against *S. aureus* for eggs in shell. The population of *S. aureus* reduced 1.8–2.5 Log CFU ml^{-1} using cold plasma

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produced by argon. They also reported no undesirable changes in physicochemical characteristics of eggs samples. Furthermore, Tian *et al*. (2010) revealed that *S. aureus* can be inactivated by APP in an aqueous environment.

Concerning the diversity of cell responses to plasma treatment, statistical analyses showed that *L. innocua* was significantly more sensitive than *S. aureus* to APP treatment (p<0.05) (Table 2).

Discussion

The present study showed that treatment with argon plasma significantly lowered the cell count of *L. innocua*. Rod *et al*. (2012) reported the effectiveness of treatment with argon cold plasma for 2 and 60 s, especially when mixed with oxygen, on the inhibition of *L. innocua*. Additionally, there have been some studies indicating the positive impacts of helium plasma on decontamination of different strains over long time periods (Song *et al*., 2009; Korachi *et al*., 2010). Besides this, the present study revealed that the longer the time of plasma treatment, the more the growth reduction of *L. innocua* occurred. The direct relationship between exposure time and antimicrobial efficiency of APP was corroborated by Ulbin-Figlewicz *et al*. (2015).

Only argon plasma at highest time point (e.g. 10 minutes) could significantly reduce the growth of *L. innocua*. Also, argon APP had the higher potential to manage *L. innocua*-induced contamination than helium APP. Applying low-pressure plasma treatment, Ulbin-Figlewicz *et al*. (2015) indicated that the logarithmic reduction value varied depending on treatment time and gas type. In contrast to our findings, they demonstrated the highest inactivation efficiency of helium plasma treatment. Cold plasma is a high energetic gas containing electrons, UV photons, ions, radicals, and excited molecules that can initiate various chemical reactions via breaking covalent bonds. These events cause serious damages to DNA, protein, lipid, and other sensitive molecules existing in the membrane of microorganisms (Kim *et al*., 2011). Thus, based on the finding of the present study, it might imply that argon APP had a greater content of free radicals than helium one. The contribution of high content of free radical was highlighted by Kim *et al*. (2011) and Noriega *et al.* (2011), whose studies reported that APP treatment with helium/oxygen mixture was more effective than single helium gas plasma. Other possible explanation might be attributed to voltage difference between two gas supplies (22 kV (argon) versus 12 kV (helium)). Niemira and Sites (2008) found out that high voltage cold plasma (15-20 kV, 60 Hz AC power) notably diminished the viability of *Salmonella* Stanley and *E. coli* O157 cells over short time periods. Feng *et al*. (2009) and Kim *et al*. (2011) reported the same results in their studies, as well. Noriega *et al*. (2011) observed that higher values of voltage led to greater decontamination potential of ACP in a model of *L.*

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innocua inoculated onto chicken muscle.

The findings of this study revealed that either argon or helium APP prevented the growth of *S. aureus* but at different conditions. Dobeic *et al*. (2016) documented the beneficial effects of non-thermal plasma treatment against *S. aureus* for eggs in shell. The population of *S. aureus* reduced 1.8–2.5 Log CFU ml^{-1} using cold plasma produced by argon. They also reported no undesirable changes in physicochemical characteristics of eggs samples. Furthermore, Tian *et al*. (2010) revealed that *S. aureus* can be inactivated by APP in an aqueous environment.

In this study, it was found that *L. innocua* showed significantly more sensitivity than *S. aureus* to APP treatment. Consistent with our findings, Han *et al*. (2016) applied ACP on *L. monocytogenes*, *E. coli*, and *S. aureus*. They observed a significant reduction in the cell viability of all pathogens, with *L. monocytogenes* showing the lowest cell survival after the treatment. Moisan *et al*. (2002) demonstrated that cold plasma involves UV radiation and erosion processes, which can inactivate the DNA and RNA or induce erosion of the micro-organism through etching. Atmospheric plasma decontamination utilizes different gas mixtures (i.e. Ar, H_2, O_2, N_2) (Moisan *et al*., 2002; Takamatsu *et al*., 2014). According to the supply gas, a variety of chemically reactive species is produced, which, in turn, exerts different influences on microorganisms (Fricke, 2012; Takamatsu *et al*., 2014). Radicals could induce erosion of the cell wall and cell membrane, as well as oxidation of proteins, DNA, RNA, and enzymes whereas charged particles affect etching, perforation of the cell wall, and electroporation (Fricke, 2012). The operating pressure mainly contributes to the presence of UV radiation in the plasma. Low pressure plasma can generate UV radiation with influential wavelengths between 200–290 nm (Munakata *et al*., 1986). Laroussi (1996) has indicated that UV radiation is not the paramount species responsible for the sterilization process at atmospheric condition. More to the point, Choi *et al*. (2006) and Laroussi (1996) have corroborated that APP fails to produce any UV radiation below 290 nm. Herrmann *et al*. (1999) exposed *Bacillus globigii* to APP operating in helium/oxygen mixtures while only UV radiation was allowed to be in contact with the spores. They reported almost no significant decline in the number of microorganisms after treatment. Birmingham (2004) has shown that the deactivation of the bacterial spore as a result of plasma does not depend on the UV radiation. Stoffels *et al*. (2002) has revealed that APP can emit UV with the highest intensities between 305 and 390 nm however damage to cells at these wavelengths is limited. In general, there have been conflicting reports in support of the possible role of UV radiation in plasma sterilization at atmospheric pressure (Park *et al*., 2003; Heise *et al*., 2004; Lee *et al*., 2005; Boudam *et al*., 2006). On the other hand, it was indicated that atmospheric argon plasma generated larger amount of

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radicals (i.e., H and OH radicals) than helium plasma (Takamatsu *et al*., 2014). Kostov *et al*. (2010) presented that ACP treatment can retard bacterial growth through the bombardment with charged particles. Although the accurate mechanisms underlying the antimicrobial impact of APP on microorganisms are still unclear, some scholars in their studies on bacteria have disclosed two influential processes: (1) the chemical etching that occurs following the interaction of reactive neutral species triggered by quite high-voltage supply (for instance 20 kV); (2) the physical damages of the bacterial membrane through the ion bombardment as well as charge accumulation (Choi *et al*., 2006; Ma *et al*., 2008; Stoffels *et al*., 2008; Kostov *et al*., 2010).

The results of this study showed that ACP with argon and helium gas flows can successfully reduce the growth of *L. innocua* and *S. aureus* inoculated on the surface of RTE fish samples. Treatment with argon plasma was observed more effective as an antimicrobial approach than when helium plasma has been used. *L. innocua* and *S. aureus* presented significantly different sensitivity to APP. Moreover, longer exposure time resulted in higher growth inhibition. Accordingly, plasma can be applicable to the decontamination measures taken against *S. aureus* and *L. monocytogenes* in RTE fish products.

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