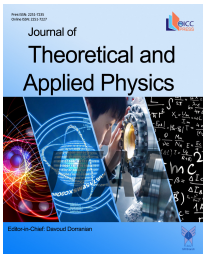

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Evaluation of surface dielectric barrier discharge (SDBD)-plasma activated normal saline for reduction of *Escherichia coli* populations in fish (*Oncorhynchus mykiss*) fillets

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Original Research

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Abstract:

Recently, the antibacterial effects of plasma-activated liquids have been noticed by food industries and researchers. Some research reported that reactive oxygens and nitrogen species (RONS) that are generated in the treatment liquids destroyed cells. In this study, we evaluated surface dielectric barrier discharge (SDBD)-plasma for inactivation of *Escherichia coli* and quality attributes of fish meat. For this aim, at first normal saline (NS) solutions activate by SDBD-plasma in the different irradiation times. Then, bactericide effects of these activated normal saline (NS*) solutions were used for inactivation bacteria, at a 5×10^7 CFU/mL suspension concentration, in the different immersion treatment times. Results show that with increasing plasma irradiation time, the concentration of RONS increased, but no significant difference was observed in its bactericide effects with increasing immersion times. Also, our result showed that discharge of plasma at 5 minutes and immersion time of 15 minutes, contained the highest inactivation of *E. coli* or bactericide effects. One of the reasons for these phenomena may be the increase of reactive species concentrations, which are poison effects on the cells. The pH, humidity, and surface color of the fish fillets were changed, but the tissue, protein, and lipid content were not changed significantly.

Keywords: Atmospheric pressure plasma; *Escherichia coli*; Fish fillets; Meat quality; Plasma-activated liquid; SDBD

1. Introduction

Nowadays, research on sterilization (killing of life) and inactivation of harmful microorganism methods are on the rise [1]. Atmospheric pressure plasmas, as one of these methods, have been developed for the medical-care industry and hospitals, as a part of the prevention of infection and sterility assurance systems [1–6]. Before studies showed that plasma, as the fourth state of matter, is an ionized gas capable of producing reactive species of oxygen, nitrogen, free radicals, electrons, ions, electromagnetic radiation, and neutral particles [4]. These reactive species react with microorganisms by destroying the cell wall or damaging DNA, or altering cell metabolism, leading to the sterilization and inactivation of microorganisms [5].

Atmospheric plasmas are produced in a variety of configurations such as plasma needles [6], atmospheric pressure plasma plumes [7], plasma jets, and microplasma jets [8–10], plasma brush [11], glow discharge torch [12], microwave plasma torch [13], and dielectric barrier discharges [14–16].

The use of atmospheric plasmas evaluated for protecting foods from biological damage-induced corrosion has become of vital importance at present [4, 17]. Because of the limitation of one of the conventional methods, the inactivation of microorganisms by plasma-activated liquids such as plasma-activated water (PAW) has attracted interest from researchers [18–21]. The idea that plasma-activated liquids contain effective performance against a wide range of microorganisms without adversely affecting the quality of the products has been verified [18–21]. Otherhands, *Escherichia coli* (*E. coli*), is a gram-negative bacteria cell found in the environment, foods, and intestines of animals and humans, which some of their strains can cause infections and illness in humans and animals [22, 23]. This bacteria were used for bactericidal studies.

In this study, we aimed to investigate the inactivation of *E. coli* and the quality attributes of the fish fillets in the washing method using the plasma-activated normal saline. The samples were immersed in chilled sterile water (denoted by Shem, as the control group), NS (as the control group), and NS* (activated by SDBD) for the immersion times of 5, 10, 15, 20, and 25 minutes. The SDBD plasma irradiation times were 5, 15, and 25 min for activating the NS. The bactericidal efficacy, pH, Lipid, protein, humidity, color, and texture (tissue) profiles were followed at 0 and 7 days after treatment.

2. Materials and methods

2.1 Preparation of bacterial strains

Lyophilized bacterial strain vials of *Escherichia coli* (ATCC: 35218) prepared from the microbial collection of Pasteur Institute of Iran. This strain, which is a negative gram and a clinical significance, was added 0.5 mL of brain heart infusion (BHI) media solutions into bacterial vials and so incubated at 37 °C overnight. Whole cultured BHI inoculated into Luria-Bertani (LB) agar (including 10 g, Bacto-Tryptone; 5 g, Yeast extract; 10 g, NaCl; $15-18$ g, Agar per liter distilled water /dH_2O), which was described by Sohbatzadeh et al. [1]. One loop from these negative bacteria was inoculated to 15 mL liquid LB (LB without agar) and incubated at 37 °C overnight. One mL of bacteria was inoculated into 15 mL liquid LB and cultured until 0.5 McFarland ($OD_{600\,nm} = 0.5$) standards corresponding to about 3×10^8 colony-forming units (CFU)/mL, which is same as CFU/cm^3 for *E. coli*. Then juvenile cultured bacteria suspend by liquid LB up to 5×10^7 CFU/ml (or $OD_{600\,nm} = 0.25-0.35$) concentration. All bacterial suspensions were performed by this method and used for sterilization by SDBD-Plasma Activated normal saline.

2.2 SDBD devise and plasma discharge

The SDBD structures were prepared based on Sohbatzadeh et al. [24] methods, which are shown schematically in Fig. 1 Our plasma source is constituted of two aluminum electrodes, which are arranged edge to edge on the top (E shape strip form) and down (U shape strip form). All of the parallel strips were $180 \times 20 \times 4$ mm dimensions. These strips, as powered electrodes, adhered to a 4 mm thick glass dielectric. The peak-to-peak applied voltage on the electrodes was 6.75 kV AC with 6.2 kHz. On the upper surface of the SDBD structure, wide and thin strips of Kapton tape were glued as a strong dielectric to prevent corona and loss of electrical power. The power of the SDBD zone was 4.2 W. Air gas was used as the carrier gas in the plasma reactor. Optical emission spectroscopy was taken beneath the surface discharge and was shown in Fig. 1(e).

Normal saline (NS), as an isotonic solution, was prepared by dissolving 9 g NaCl in one liter of H_2O. It was sterile by autoclave (1.5 At, 125 °C, 15 minutes). About 10 mL NS was activated under SDBD gas circulation for different times 0 (mean that NS without plasma discharge, as a control), 5, 15, and 25 minutes in the glass petri-dish. In this paper, SDBD-plasma-activated normal saline, in the 5, 15, and 25 min. were shown by NS^*_5, NS^*_{15}, and NS^*_{25}, respectively. About to 5×10^7 CFU/mL treated by each of these activated NS (NS^*_5, NS^*_{15}, and NS^*_{25}) at the different immersion (0, 10, 15, 20 and 25) times.

2.3 Chemical parameters assay

To determine the concentration of NO_3^-, NO_2^-, and H_2O_2 in NS* solutions, UV absorption spectroscopy was performed following the methods described by Liu et al. [16]. To detect the absorption spectra of RONS in NS a conventional double-beam UV-Vis spectrophotometer (PG Instruments T80+, UK) was used. A quartz cuvette with a standard optical path was used to place the samples in the spectrophotometer and measure the absorption spectra of RONS in discharged NS. The RONS concentration was determined from absorbance (Abs) spectra by using the Beer-Lambert law: $\mathrm{Abs}(\lambda) = \varepsilon LC$, where ε is the molar absorptivity of the chemical species at a certain wavelength λ, L is the optical path length (which is one centimeter), and C is the RONS concentration.

2.4 Fish's meat preparation

All of the fishes (*Oncorhynchus mykiss* species) with 800 ± 150 g in body mass, were purchased from the Mahmudabad beach market (North of Iran). The "fish fillets" were cut into slices at the same weight ($\sim 4 \pm 1$ g) and size ($\sim 20 \times 15 \times 20$ mm) using a meat slicer. All samples were placed on a clean bench, and all examinations were performed in triplicate.

2.5 Fish meat washing treatment and bacterial enumeration

The chilled "fish fillets" slice samples were immersed in 15 mL of chilled sterile water (as a Shem), NS, and NS*, which each contains 5×10^7 CFU/mL of fresh and juvenile *E. coli* (with OD_{600} nm $= 0.25-0.35$ scales). Inoculated samples were incubated at 4 °C with 100 rpm at different times 0 (as a blank), 10, 15, 20, and 25 minutes. After these times about 100 μL of each sample, was diluted in 1 mL of liquid LB, and 100 μL of this dilution was spread on the solid LB agar plates in triplicates. Plates were incubated at 37 °C for 12 hours, and so CFU was determined.

2.6 Quality analysis of fish meat

For instrumental color measurement, we measured the surface-color values by the international commission on

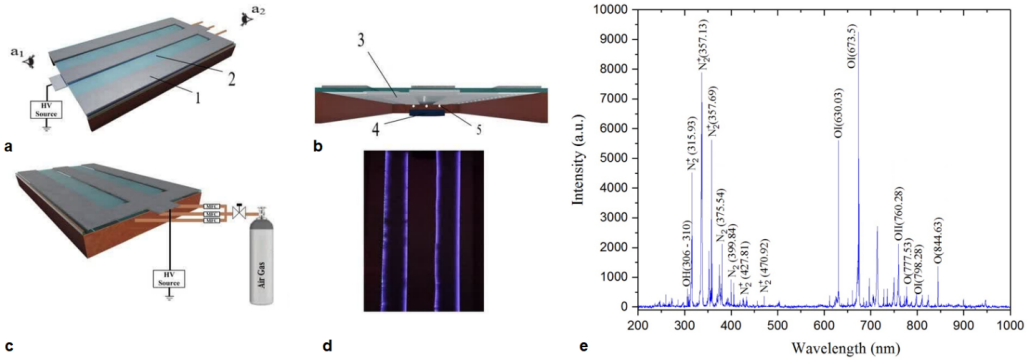

Figure 1. Schematic diagram of SDBD device, and highlighted spectral lines of the plasma discharge. (a) Whole view. (b) View from a1 side. (c) View from a_2 side. (d) Produced parallel lines of plasma between the electrodes. (e) Highlighted spectral lines of the plasma discharge. Numbers showed in the a, b, and c including: 1= upper electrode, 2= Dielectric barrier, 3= Lower electrode, 4= Sample, 5= Gas inlet; HV= High voltage power supply, MFC = Mass flow controllers.

Illumination (CIE) standard. Based on Values L* (lightness, score 0 - 100), a* (redness+, greenness–), and b* (yellowness+, blueness–) of the samples by using a colorimeter (Colorimeter, IMG-Pardazesh, CAM-System XI, Iran).

An oven (Oven, UF55-Memmert, Germany) and a digital scale (Digital scale, GF600-AND, Japan) were used to measure the moisture content (%). For measurement of the pH, protein, and lipid contents, at first, samples were homogenized by ULTRA-TURRAX Homogenizer T25 (IKA Co., Germany). The pH was measured using a pH meter 3503 (Jenway Co., England), protein contents were measured using a Kjeldahl system V50 (Bakhshilab Co., Iran), and lipid content was measured using a Soxhlet extractor system 6CTF (Bakhshilab Co., Iran). The texture (tissue) analysis, treated, and untreated fish meat samples were measured by a texture analyzer TA (Koopa Co., Iran). The values obtained for hardness, rigidity, adhesive force, resilience, stringiness length, and fracturability were recorded. Three replicate samples were used for each treatment.

2.7 Statistical analysis

Statistical analyses were carried out using SPSS ver. 19.0 software. The normality of data was tested using the Shaprio-Wilk test. Datasets were analyzed using a general linear model (GLM) with Turkey's pairwise comparison to assess the significant difference between groups. The statistical significance levels were set at $p < 0.05$.

3. Result and discussion

Evaluating the induced concentration of the RONS in NS is essential to clarify which one of them could be more responsible for the inhibition of the bacteria inactivation. Optical emission spectroscopy (OES) enables analysis of the radiation emitted from atoms, ions, molecules, and radicals when they are excited by an electric field or by collisions with other particles. These emission lines and bands are very useful for diagnostic purposes because they impart important information about the plasma. Fig. 1(e). displays the emission spectra from 200 nm to 1000 nm and clearly discloses the existence of excited species in SDBD plasma that activated NS. Some highlighted spectral lines such as reactive atomic oxygen and nitrogen as products of the SDBD were marked on the spectra. Although the relative concentration of the OH seems to be low, the concentration of reactive OH grows significantly due to the collision between air, humidity, and meta-stable excited argon.

To confirm the formation of NO_3^-, NO_2^-, and H_2O_2 inside the NS, we conducted the UV absorption method. Fig. 2(a-c) show the concentration of the RONS in the NS*. The levels of NO_3^- in treated samples were significantly higher than the other species and the increasing rate of the NO_3^- concentration during the treatment was faster. The NO_3^- concentration after SDBD treatment for 5 min was 39.60 mg/L, which was risen to 101.73 mg/L with a treatment time of 25 min. The concentration of NO_2^- was lower compared to NO_3^-. After 25 minutes of plasma irradiation, the concentration of NO_2^- reached 71.74 mg/L, which was lower compared to NO_3^-. Finally, the concentration of H_2O_2, which was the lowest compared to other species, reached 32.18 mg/L after 25 minutes of plasma irradiation.

Type of discharge, running gas, humidity, voltage, the distance between electrodes, etc., determine the chemical species to be generated in plasma-activated solution. It was expressed that plasma-activated solution consists of various RONS, such as O, OH, O_3, H_2O, NO, NO_2^-, NO_3^-, and ONOO- that are responsible for microbial inactivation [25]. Some articles reported that RONS play a significant role in bacterial- and antifungal inactivation [26, 27], whereas other research groups proposed that ONOO- is the important species for bacterial inactivation [28, 29]. Furthermore, it was reported by others that NO_2^- / NO_3^- cause bacterial inactivation [30, 31].

Air as a running gas is low-cost yet highly effective source of oxygen and nitrogen. A continuous flow plasma reactor enabled the effective and rapid transfer of plasma-generated chemistry from gas to liquid phases. Negative and positive ions were generated by energetic electron ionization of water molecules at the gas-liquid interface. These reactive species can be divided into short- and long-lived ones [32–34]. The positive and negative ions reacting with water molecules can generate various radicals and result in cell death [35].

Many researchers depicted that plasmas have been known to have great potential to inactivate microorganisms due to

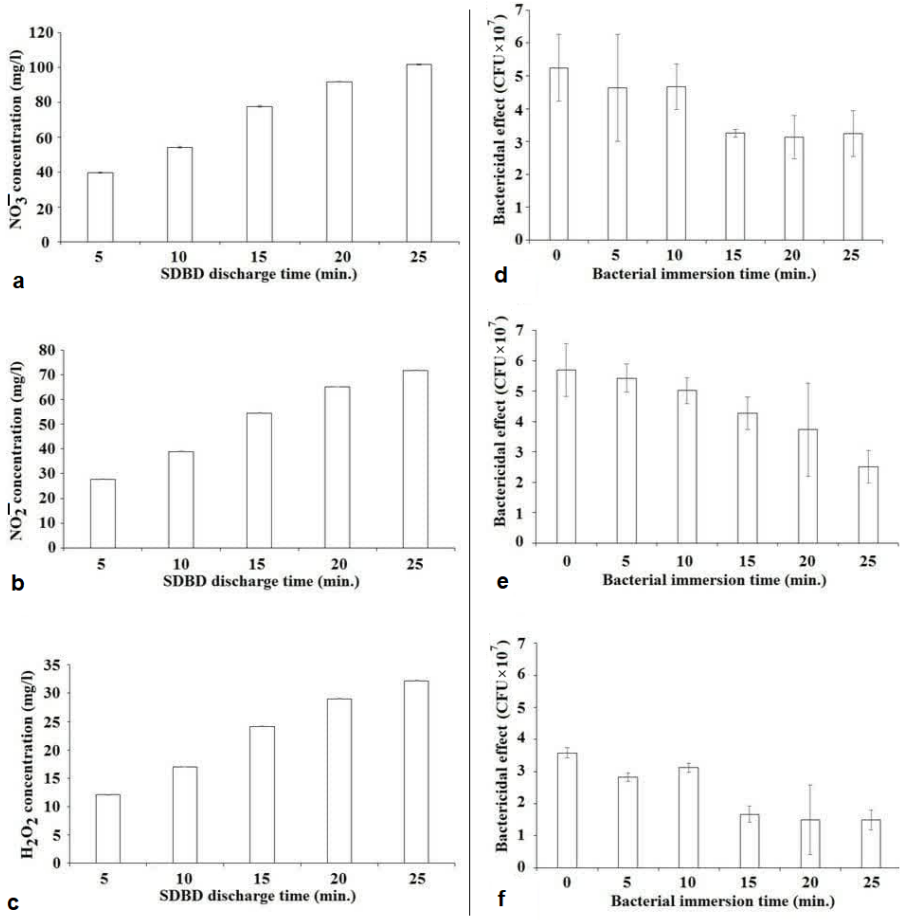

Figure 2. Concentration of NO_3^-, NO_2^-, and H_2O_2 after plasma treatment in SDBD-plasma activated NS and *E. coli* log reduction in different plasma treatment time and bacterial immersion time. (a) NO_3^- concentration after 5, 10, 15, 20 and 25 min plasma treatment. (b) NO_2^- concentration after 5, 10, 15, 20 and 25 min plasma treatment. (c) H_2O_2 concentration after 5, 10, 15, 20 and 25 min plasma treatment. (d) Log reduction of *E. coli* in NS^*_5 in the different bacterial immersion times. (e) Log reduction of *E. coli* in NS^*_{15} in the different bacterial immersion times. (f) Log reduction of *E. coli* in NS^*_{25} in the different bacterial immersion times.

their simple and inexpensive designs, non-toxic nature ease of mild and ambient operation conditions, whose heat or UV is harmless to living cells [36]. Several techniques have been employed to generate plasmas at atmospheric pressure. The general advantage of these techniques is that they allow the formation of an abundance of active species and can be operated close to ambient temperature [37, 38].

Fig. 2(d-f) show the *E. coli* log reduction in different plasma treatment times and bacterial immersion times for plasma-activated NS. The results show that 5 min discharge of plasma and 15 min for immersion time resulted in the highest inactivation of *E. coli*. The results show that with plasma irradiation for 5 minutes and with different immersion times, with the increase of immersion time, the inactivation of microorganisms takes place more effectively. When the plasma irradiation time was increased to 15 and 25 minutes, no significant difference was observed in the results related to the inactivation of microorganisms in different immersion times with increasing immersion time. In 15 minutes, the bactericidal effect was less than 5 minutes of plasma radiation. However, in 25 minutes of plasma irradiation, the bactericidal effect increased again and it was comparable to the first case, but with the difference that in this case the bactericidal effect was no longer increased and it was almost the same at all immersion times. Therefore, it can be concluded that in 5 minutes of plasma irradiation, the bactericidal effect was related to the presence of active species and their effect on microorganisms, but with increasing time due to the increase in the concentration of NO_2^-, NO_3^-, and H_2O_2, the bactericidal effect was related to their presence and it is poisoning the environment.

3.1 Comparison of fish's meat quality

In the before plasma-activated solution bactericidal studies, the lack of isotonic solution (solutions that contain the same concentration of water and solutes as the cell cytoplasm) is an important disadvantage. Because, we don't know the number of inactivated microorganisms, killed by the turgescence effects. Our idea is that we use Shem groups as a control, for the evaluation of inactivated microorganisms and quality attributes of products by NS (an isotonic solution) and NS* solutions. The use of Shem groups or "treatment by distilled water" as a hypotonic solution for bacterial life is missed and minimized this defect. The

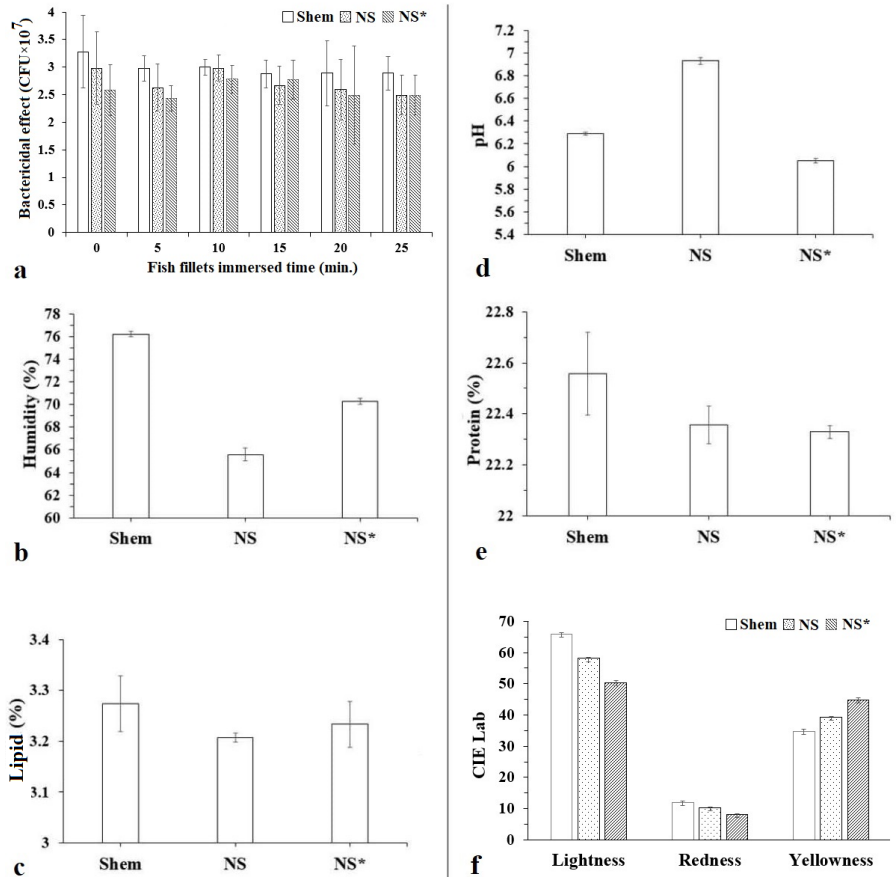

Figure 3. Comparison of bactericidal effect on fish fillets and fish's meat quality: (a) Comparison of bactericidal effect on fish fillets in different immersed times. (b - e) showed humidity%, pH, lipid%, and protein% of fish's meat (~ 0.5 g) after immersion in the shem, NS, and NS*, respectively. All of the analysis of humidity, pH, lipid, and protein content was performed on 0.5 g meat, which was homogenized in one milliliter by homogenizer. (f) Surface-color values of fish meat samples in Shem and after washing with NS and NS* samples after 7 days.

chilled "fish fillets" slice samples were immersed in 15 mL of chilled sterile water (as a Shem), NS, and NS* after different times (0, 5, 10, 15, 20, and 25 min). The comparison of the bactericidal effects of these solutions showed that there is no significant difference (Fig. 3(a)).

To evaluate the effects of washing on the moisture content (%) of samples, the changes in these values were analyzed. The moisture content (%) values were significantly decreased after 7 days (Fig. 3(b)). Results show that the use of NS* compared to NS causes the amount of moisture to decrease to a lesser extent after seven days, which can help the freshness of the meat and its shelf life for a longer time. The water-holding capacity of meat depends on storage conditions, freezing, frozen storage, and thawing [39]. Considering the same conditions for the samples, it can be concluded that the presence of active species in NS* can maintain moisture content (%) in the samples. Therefore, using NS* in washing can be a suitable option to preserve the moisture level in the samples for a longer time.

The pH value changes in Shem and samples treated with NS and NS* are shown in Fig. 3(c). The initial pH value was about 6.29 at the end of storage. The initial post-mortem pH of fish varies with species, catching ground, and season [40]. pH decreased after treatment with NS*, while it increases in the samples treated with NS. pH increases slightly because of the formation of alkaline compounds. In samples treated with NS, due to the fact that NS has less antibacterial properties than NS*, the increase of pH throughout 7 days of storage might be attributed to the formation of basic decomposition products, such as ammonia and trimethylamine. These compounds are produced by endogenous enzymes and bacterial spoilage [41]. The increase and decrease of pH in the samples treated with NS and NS* can be caused by the increase of alkaline compounds such as ammonia by the bacteria causing fish spoilage [40]. On the other hand, according to the bactericidal properties of NS*, it is possible to justify the reduction of pH in the samples. In the comparison of lipid and protein content (%), all of the samples with NS and NS* treatment exhibited no obvious changes (Fig. 3(d-e)). The appearance of meat, in particular, color as an indicator of the freshness and quality of meat, affects consumers' meat-purchasing decisions more than any other trait related to meat quality [42]. Thus, the changes in the surface color values of the fish meat samples that were washed with NS and NS* were measured. The results showed that after 7 days, the L* values and a* values of samples were decreased from those of the unwashed samples, while the b* values increased ($p < 0.05$) (Fig. 3(f)).

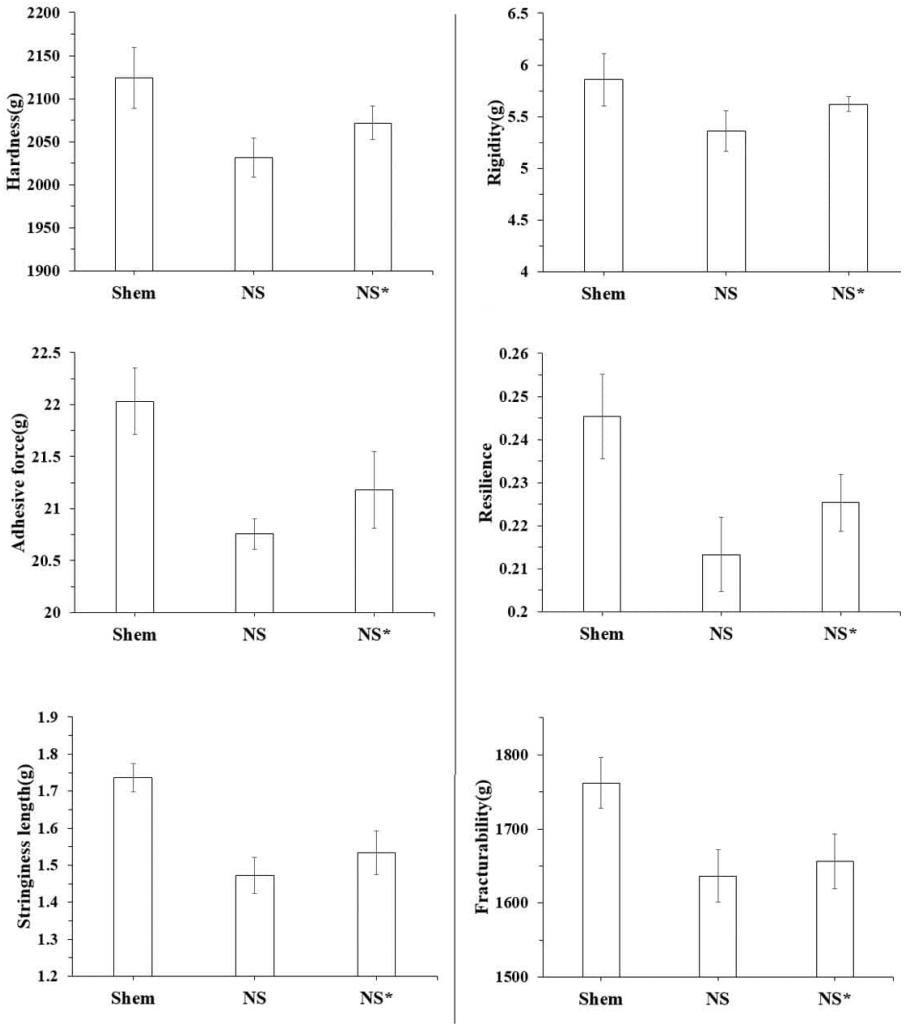

Figure 4. Tissue-profile analysis of fish meat samples after treatments with NS and NS*.

Similarly, Ulbin-Figlewicz et al. [43] determined that the L* values and a* values in pork loin decreased substantially following treatment with low-pressure cold plasma, while the b* values increased. By contrast, Fröhling et al. [44] determined that the L* value of porcine muscle was not strongly affected by indirect plasma treatment but slightly increased. For a* value and b* value, our results were in agreement with previous studies [42], while in some articles, results contrary to our results were observed [45]. This difference may be caused by the different processing parameters and the meat types.

Fig. 4 presents the effect of washing fish meat samples with NS and NS* on the texture parameters after 7 days. The results indicate that no texture parameter was affected significantly ($P > 0.05$) by the NS and NS*. Our results are in agreement with the results of previous studies [40, 46]. Similar to previous studies [42, 47, 48], our results showed that plasma discharge cannot cause tissue parameters (including: hardness, springiness, cohesiveness, gumminess, and chewiness) exchange. Also, the use of air-plasma has shown that plasma treatment does not influence the tissue products [46, 47, 49, 50]. Thus, the method that we used in this study could serve as a non-destructive washing method.

4. Conclusion

Our results showed that SDBD plasma-activated NS in atmospheric-pressure air is proved to be a promising disinfectant for *E. coli* and could be a useful scheme in increasing the shelf life of fish meat. The physicochemical attributes of the fish meat samples were similar to those of samples treated with NS and only the color of the samples had significant changes. Therefore, this plasma device must be improved to minimize the adverse effects on the physicochemical and sensory qualities of meat. From the results, plasma-activated NS as proposed in this research presents an attractive method of producing plasma-activated solutions for biological applications that cannot be easily reproduced using chemical means alone.

Authors Contributions
Meysam Nikpour and Mohammadreza Hosseinzadeh performed experiment and wrote the draft, Saeed Mirzanejhad, Abasalt Hosseinzadeh Colagar and Farshad Sohbatzadeh conceived, designed the experiments and reviewed.

Availability of data and materials
The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of Interests
The author declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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