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Effects of the Cold Atmospheric Plasma Treatment Technology on *Staphylococcus Aureus* and *Escherichia Coli* Populations in Raw Milk

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ABSTRACT

Today, various sterilization methods are used for the removal of microorganisms, some of which are based on thermal methods that have negative effects on the physicochemical properties of milk. The present study aimed to investigate the effects of cold plasma at atmospheric pressure on the population of *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) in raw milk. Initially, a plasma jet filled with argon gas was used to evaluate the antibacterial effects of cold plasma. Following that, pasteurized milk samples (1.5% and 3% fat) were infected with standard strains of *E. coli* and coagulase-positive *S. aureus* and irradiated with cold plasma at the frequency of 22, 28, and 33 kHz and voltage of 20, 12.5, and 10 kV for five minutes. The results of statistical analysis and Tukey's test indicated that the *E. coli* and *S. aureus* microbial load was significantly lower in the 1.5% fat milk compared to the control group ($P<0.05$). In addition, the milk samples exposed to plasma at 20 kV and 28 kHz showed the most significant reduction in the number of *E. coli* bacteria compared to the control samples ($P<0.05$). The milk samples exposed to 10 kV and 33 kHz also showed the most significant reduction in the *S. aureus* microbial load. According to the results, cold plasma could decrease the microbial load of milk containing 1.5% fat more significantly compared to the 3% fat milk. Therefore, plasma could be a proper alternative to thermal decontamination methods for raw milk. However, its application requires further studies to determine the intensity and duration of the exposure of microorganisms to cold plasma at atmospheric pressure.

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Introduction

According to the World Health Organization (WHO), foodborne illnesses are a major public health concern across the world (1-3). Milk spoilage due to contamination with microorganisms is an important cause of hospitalization due to food poisoning. The number and type of milk microorganisms and dairy products depend on the quality of raw materials, production conditions, temperature, and shelf life (4).

Rod-shaped gram-negative bacteria and various species of *Streptococcus*, molds, and yeasts are the main causes of milk spoilage. Today, various sterilization methods are used to remove microorganisms, some of which are thermal-based disinfection approaches, such as steam sterilization, autoclave, and heating. The thermal-based disinfection technology has side-effects on the nutrients and sensory and functional properties of food (5, 6). Thermal methods are costly and cannot eliminate the microbial load of milk (7). During thermal processes, the structure of milk and its organoleptic properties change, thereby leading to the loss of its efficiency in the production of other dairy products. In several cases, the resulting products do not acquire customer satisfaction (8).

The plasma technology is a new method of microorganism destruction used in the food industry. Plasma is the fourth state of matter in the form of ionized gas, which could be generated by electrical discharge (9). Electrical discharge at atmosphere pressure and low temperature has made this process practical, cost-effective, and suitable for the products in which the thermal process is not desirable (10). The plasma state contains a collection of positively or negatively charged ions, electrons,

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neutral/charged gas molecules, radicals, and other particles (11). Atmospheric plasma is divided into two categories of non-thermal plasma and thermal plasma depending on the production method. Non-thermal plasma is referred to as 'cold plasma' or unbalanced plasma due to its energy level, temperature, and ion density. The most prominent feature of cold plasma is its efficiency in decontamination as it exerts no debilitative effects on the decontaminated surface due to the weak nature of the partially ionized gas (12). The antimicrobial effects of cold plasma depend on the type and technology of the plasma, such as the energy level used to produce the plasma, mixture of the working gas, intensity and duration of sample exposure, system design, and the flow rate and pressure (13).

The source of atmospheric energy production could be microwave, radio frequency (RF), pulsed electric field, alternating current, and direct current. The main devices used to produce plasma include corona discharge, micro hollow cathode, dielectric plasma needle/pen, and atmospheric pressure jet. Pressurized atmospheric jets are the most widely used devices in industries such as lighting and surface decontamination. Inactivation of microorganisms during plasma treatment results from the direct contact of the active antimicrobial species with charged particles (14). Accumulation of the charged particles on the surface of the cell membrane may rupture the cell membrane. Oxidation of lipids, amino acids, and nucleic acids through reactions with oxygen and nitrogen causes changes in these compounds, thereby leading to the death or damage of microbial cells. In addition, UV photons and passive particles could cause changes in the DNA of microorganisms, thereby impairing cell proliferation depending on the characteristics of the plasma, such as the voltage, applied gas, the water content in the gas, the distance of the microorganism from the discharge surfaces, and the type of microorganisms (gram-positive, gram-negative, spores, and other species) (15).

A major issue with pasteurized milk is that in addition to the reduction of vitamin C, vitamin B1 (thiamine) is also lost during the pasteurization process due to temperature sensitivity (~10-35%) (16). Decreased vitamin B2 (riboflavin) has also been observed in small doses. Heat treatments have a significant effect on milk flavor and cause different profiles in milk properties (17).

In the present study, we used modern non-thermal methods (e.g., cold atmospheric plasma) to produce foods with the least number of pathogens that have advantages such as the significant reduction of waste and no environmental pollution, lower energy consumption compared to thermal methods, and reduction of the microbial load. In order to achieve the optimal quality of raw milk and provide products with proper microbial properties to the consumer market, the present study aimed to use the cold atmospheric plasma method as an alternative to non-thermal pasteurization methods. This could be a new step in the food industry of Iran since no research has been focused on the non-thermal pasteurization of milk (both low-fat and high-fat) using cold atmospheric plasma in Iran. Our findings could illustrate a proper alternative to currently used pasteurization methods.

Materials and Methods

Atmospheric Cold Plasma Generator

In this study, we used an atmospheric pressure plasma jet (JET; High Tech Company, 00245, Tehran, Iran) filled with argon gas. In terms of the electrode structure, the device was designed and fabricated with two parallel cylindrical copper electrodes (height: 0.12 mm, diameter: 45 mm), and the electrodes with dielectric plates were covered with glass (thickness: 1 mm). The distance between the electrodes was controlled by a separator. By applying an alternating voltage with a magnitude of 10 kV to the peak and a frequency of 25 kHz to the electrodes, a uniform plasma in air and a volume between the two electrodes were formed. The manual circuit consisted of a clock, MOSFET, and the transformer and was used to generate a high voltage. To induce a high voltage in the secondary winding of a transformer, a high-current wave was required to pass through the primary winding. For this purpose, am initial wave was generated with an adjustable frequency and a duty cycle by the clock and entered the MOSFET (IRFP460) to increase the current. Following that, it entered the primary winding of the transformer with a sufficiently high current, finally inducing a high voltage in the secondary winding (Figure 1).

Figure 1. A view of the atmospheric cold plasma generator.

Preparation of Milk Samples and Plasma Exposure

Initially, pasteurized milk was purchased at the market and heated for 20 minutes. Afterwards, the milk samples without a microbial load containing 1.5% and 3% fat were obtained from valid markets. *Escherichia coli* and *Staphylococcus aureus* were purchased from the Pasteur Institute in Tehran, Iran and cultured on a specific culture medium. At the next stage, 2.7 milliliters of cold sterilized milk, which had been confirmed to be free of microbial flora, was poured into test tubes, and 300 microliters of the bacterial culture with McFarland standard turbidity was added to each tube. The samples were divided into control and plasma treatment groups (18). For each bacterial strain, three replicates of the milk samples were used, and one milk sample was considered a control (Figure 2). Table 1 shows the performed plasma treatment.

Table 1: Experimental design to expose contaminated milk samples at different voltages and frequencies

Contaminated Samples	Frequency (kHz)	Voltage (kV)	Time (min)
Treatment 1	28	20	5
Treatment 2	22	12.5	5
Treatment 3	33	10	5

The milk samples exposed to cold plasma were diluted (10^{-1}-10^{-5}) and cultured. Nutrient agar and Brad Parker media were used for the culturing and isolation of *E. coli* and *S. aureus* strains, respectively. After 24-48 hours of incubation at the temperature of 37°C, the bacterial colony count per plate was determined using an automated colony counter (SphereFlash, 2016/679, 08030 Barcelona, Spain). The mean colony count per plate was determined, and the result was reported in CFU/ml based on the dilution coefficient of the sample in each plate.

Figure 2. Exposition of contaminated milk samples with cold plasma

Statistical Analysis

Data analysis was performed in SPSS version 24 and the Excel software. The obtained data were obtained from three replications. Significant differences between the measured variables were evaluated using one-way analysis of variance (ANOVA) with 95% confidence

interval, and the means were compared using Tukey's test. The results of the comparisons were presented graphically in the Excel software.

Results

E. coli Count in 3% Fat Milk

According to the results of SPSS analysis and the comparison of means using Tukey's test in 3% fat milk, the bacterial colony count of *E. coli* significantly decreased in the contaminated samples exposed to cold plasma compared to the control samples (P<0.05; 95% CI). The reduction in *E. coli* bacterial count compared to the controls was 1.2 times. However, no significant difference was observed between the bacterial population of the samples at different voltages and frequencies (Figure 3).

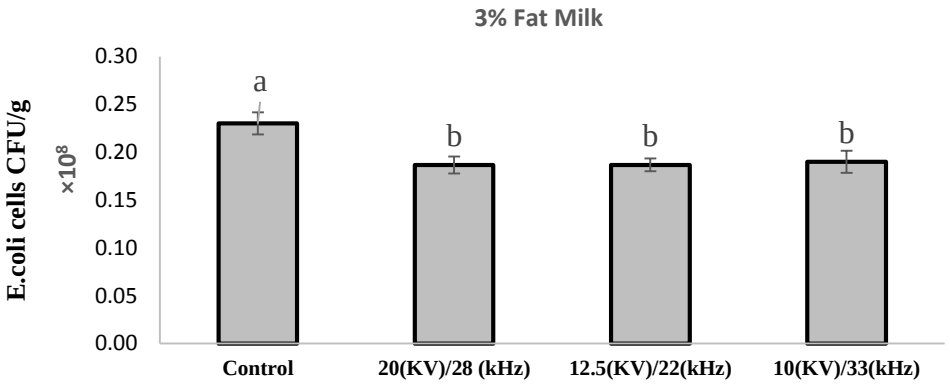

Figure 3. Colony count results of *E. coli* strains in 3% fat milk samples after irradiation of cold plasma. The numbers in each graph are the average of three iterations. The different letters shown in each column indicate that there is a significant difference between the different treatments (p<0.05).

E. coli Count in 1.5% Fat Milk

According to the results of SPSS analysis and the comparison of means using Tukey's test in 1.5% fat milk, the bacterial colony count of *E. coli* significantly decreased in the milk samples exposed to cold plasma compared to the control samples (P<0.05). However, no significant difference was observed between the 1.5% fat milk samples at voltages of 12.5 and 10 kV and frequencies of 22 and 33 kHz. On the other hand, the milk samples exposed to cold plasma at the voltage of 20 kV and frequency of 28 kHz showed a significant reduction in the *E. coli* bacterial colony count compared to the other samples and controls (P<0.05). The reduction in the *E. coli* bacterial count was twice the control samples (figures 4 & 5).

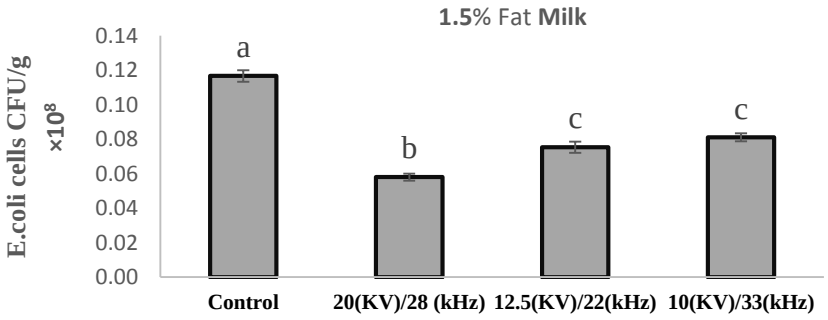

Figure 4. Colony count results of *E. coli* strains in 1.5 % fat milk samples after irradiation of cold plasma. The numbers in each graph are the average of three iterations. The different letters shown in each column indicate that there is a significant difference between the different treatments (p<0.05).

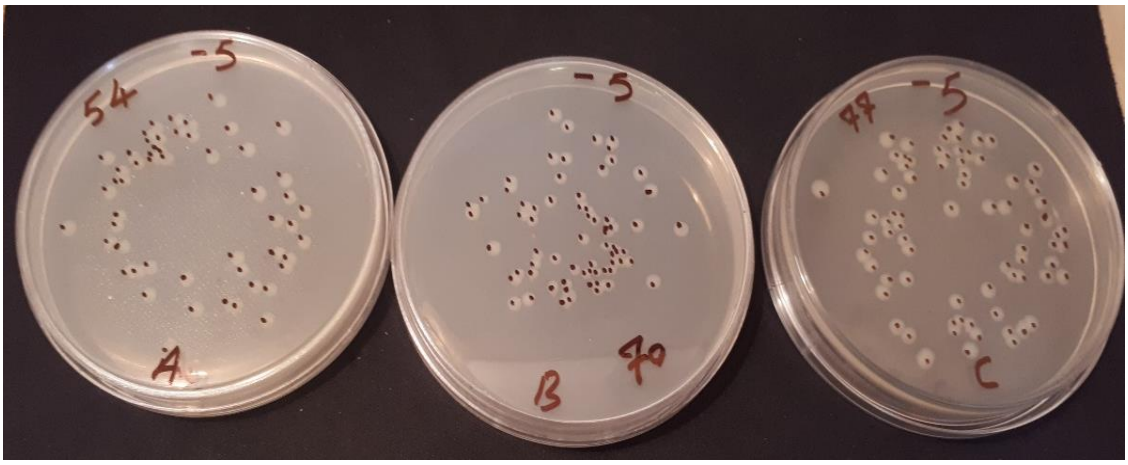

Figure 5. Results of *E. coli* counts in plasma-exposed samples. A: with 20 kV and 28 kHz, B: Plate exposed with 12.5 kV and 22 kHz, C: Plate exposed with 10 kV and 33 kHz cold plasma.

Comparison of E. coli Counts in 3% and 1.5% Fat Milk

According to the comparison of the *E. coli* bacterial counts in 3% and 1.5% fat milk using two independent sample t-tests, *E. coli* bacterial count had no significant difference between these groups (P<0.05) (Table 2). Furthermore, the results of one-way ANOVA and Tukey's test indicated no significant difference between different voltages and frequencies in 3% and 1.5% milk in this regard compared to the total means. These findings confirmed the ability of the plasma to reduce *E. coli* bacterial counts at both milk fat concentrations compared to the control samples. The results of the independent sample t-tests also indicated that since the significance (two-tailed) obtained from all the treatments was less than 0.05 (P<0.05), the 3% and 1.5% fat milk samples had no significant differences (Figure 6).

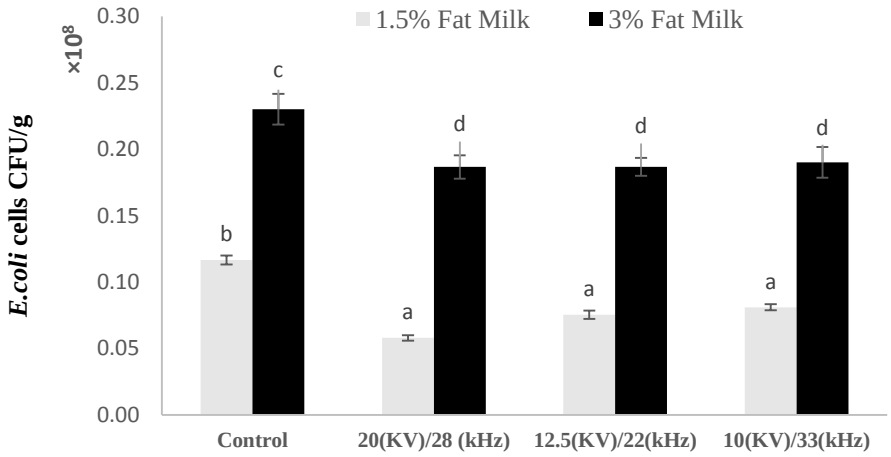

Figure 6. Comparative colony count results of *E. coli* strains in 3 and 1.5 % fat milk samples after irradiation of cold plasma. The numbers in each graph are the average of three iterations. The different letters shown in each column indicate that there is a significant difference between the different treatments (p<0.05).

Table 2. Two independent sample t-tests data in comparing 3% and 1.5% fat milk samples.

Independent Samples Test								
		Levene's Test for Equality of Variances		t-test for Equality of Means				
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
Control	Equal variances assumed	1.730	.259	-9.430	4	.001	-11333333.333333334	1201850.425154663
	Equal variances not assumed			-9.430	2.331	.007	-11333333.333333334	1201850.425154663
20(KV)/28 (kHz)	Equal variances assumed	4.238	.109	-14.199	4	.000	-12866666.666666668	906151.814604546
	Equal variances not assumed			-14.199	2.222	.003	-12866666.666666668	906151.814604546
12.5(KV)/22(kHz)	Equal variances assumed	3.301	.143	-15.073	4	.000	-11133333.333333336	738617.326872011
	Equal variances not assumed			-15.073	2.865	.001	-11133333.333333336	738617.326872011
10(KV)/33(kHz)	Equal variances assumed	2.462	.192	-9.256	4	.001	-10900000.000000000	1177568.115510379
	Equal variances not assumed			-9.256	2.160	.009	-10900000.000000000	1177568.115510379

***S. aureus* Count in 1.5% Fat Milk**

Considering the significant reduction in the *E. coli* colony counts in the 3% and 1.5% fat milk samples (Table 2), the 1.5% fat milk sample was only used for evaluating the effect of the plasma on *S. aureus* bacterial colony count. According to the results of SPSS analysis and the comparison of means using Tukey's test in 1.5% fat milk, the *S. aureus* bacterial count significantly decreased in the milk samples exposed to the plasma compared to the control samples ($P<0.05$). In addition, a significant difference was observed in the colony count of *S. aureus* at different voltages and frequencies. The reduction in the *S. aureus* bacterial colony count was twice the reduction observed in the control samples. Furthermore, the milk sample exposed to the voltage of 10 kV and frequency of 33 kHz had significantly lower counts of *S. aureus* ($P<0.05$) (figures 7 & 8).

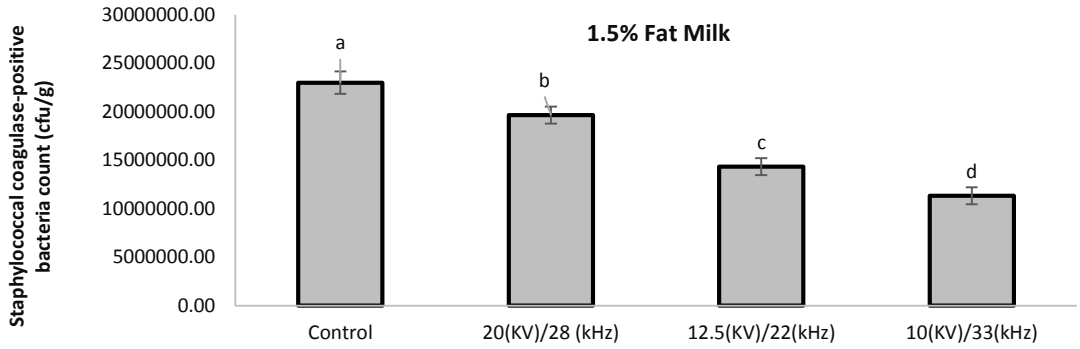

Figure 7. Colony count results of *S. aureus* strains in 1.5% fat milk samples after irradiation of cold plasma. The numbers in each graph are the average of three iterations. The different letters shown in each column indicate that there is a significant difference between the different treatments ($p<0.05$).

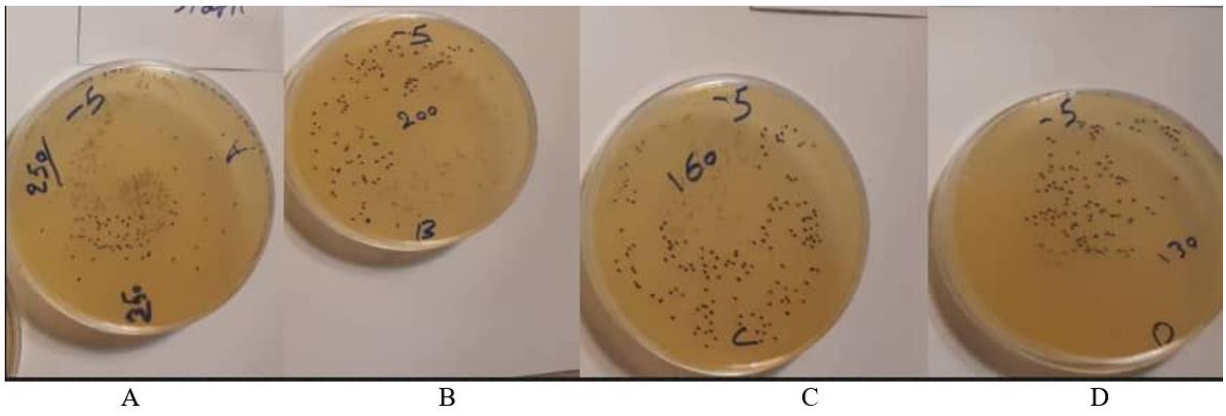

Figure 8. Results of *S. aureus* colony counts in plasma-exposed samples. A: Control, B: Plate exposed with 20 kV and 28 kHz, C: Plate exposed with 12.5 kV and 22 kHz, and D: Plate exposed with 10 kV and 33 kHz cold plasma.

Discussion

Atmospheric cold plasma is a new method used to reduce the microbial load of raw milk and other protein foods and increase the shelf life of milk and consumer health. The reduction of bacterial populations in milk could be accomplished by using the cold plasma treatment at various voltages, frequencies, times, and gases, as well as different current intensities (flows) and devices to generate cold plasma. Some of these techniques include dielectric-barrier discharge (DBD), plasma jet (JET), and radiofrequency plasma (19).

To date, few studies have been focused on the safety and quality of plasma-treated foods, proposing contradictory results. Therefore, further investigation is required in this regard as in some cases, no changes have been reported, while other studies have shown significant changes. These discrepancies could be due to experimental differences in plasma devices, while factors such as the type and characteristics of the treated foods, their water activity, and protein/fat content largely influence the disinfection process.

A study regarding the antibacterial effects of plasma on the microbial growth of *Listeria innocua* and *S. aureus* in fish products indicated that argon plasma at each time point (4, 6, and 10 minutes) had a significant inhibitory effect on the growth of the microorganisms (20). Inactivation of microorganisms using plasma is an emerging technology in the fields of food and biomedicine, as well as a significant determinant of the destruction of microorganisms by cold

plasma. In this technology, the production of ions and active forms of hydroxyl, oxygen, ozone, and hydrogen peroxide by gas ionization is the main mechanism of disinfection.

The positive effects of the plasma technology have been previously confirmed in the studies regarding the survival of *E. coli*. According to experimental results, 15 minutes of precipitation could reduce the population of *E. coli* by 100% (21). The results of another experiment indicated that using a high voltage for a long time could further reduce the load on *E. coli* (22). In addition, a study evaluating the effects of plasma on the microbial quality of cheese and ham pieces inoculated with three strains of *Listeria monocytogenes* demonstrated that the colony count of *L. monocytogenes* significantly decreased in the cheese and ham samples (23). The researchers also explored the combined effects of plasma gases in their experiment and obtained acceptable results. For instance, the reduction of the contamination of red pepper powder was assessed using cold plasma obtained from helium and oxygen gases in one of the experiments. According to the findings, the combined use of gases caused the produced plasma to prevent the growth of *Bacillus cereus* in red pepper powder (24).

Another study investigated the effects of hydrogen peroxide concentration and atmospheric cold plasma on the destruction of *E. coli* inoculated into water. According to the obtained results, the increased concentration of hydrogen peroxide and increased treatment duration were most effective in the destruction of the water microorganisms by the cold plasma (25). In several cases, the cold atmospheric plasma technology could be a substitute for conventional thermal methods. For instance, non-thermal atmospheric pressure plasma was used as an alternative to thermal pasteurization in a study to inactivate microorganisms in barberry juice, and the findings indicated no significant difference between plasma irradiation and pasteurization, while a significant difference was observed between these methods and the control group (26).

Although pasteurization and sterilization have no effect on the total fat content of human milk, sterilization may reduce the available fat content by >10%. In this regard, the findings of Fidler et al. (1998) indicated that the fatty acid composition of human milk did not change by pasteurization, while it slightly changed by sterilization (27). In the present study, the milk fat content was observed to affect the efficacy of cold plasma, which is in line with the results obtained by Senhaji et al (28), demonstrating the protective effects of fat on the heat resistance of bacteria II (28). However, the protective effects of fat would not occur in most emulsions due to the small size of the fat droplets in suspension (28).

Using the new atmospheric cold plasma technology in the dairy industry could effectively decrease the microbial load. In a study in this regard, a combination of nitrogen and oxygen gases was used with helium gas to reduce the microbial load of milk, and the obtained results showed that with the increased duration of plasma exposure, the rate of microorganism destruction increased as well (29). In another study, the effects of cold plasma atmospheric pressure by DBD at different times (three, six, and nine minutes) were investigated on the inactivation of *E. coli*. According to the findings, the increased duration of plasma irradiation was associated with the higher rate of bacterial inactivation and the reduction of milk pH, which was significantly lower in the treated samples compared to the control samples (30). In the mentioned study, the safety characteristics and microbial quality of milk were also evaluated after treatment with DBD plasma for five and 10 minutes, and the obtained results indicated that plasma treatment for less than 10 minutes could improve the microbial quality of milk with minor changes in its physicochemical properties (28).

A molecular study investigated the effects of cold plasma at atmospheric pressure on the sterilization and macromolecules of bovine milk. In the mentioned research, the cold plasma produced by the DBD was charged to milk containing *Candida albicans* colonies for one, three, six, nine, and 12 minutes. The findings indicated the presence of no fungi at nine and 12 minutes, indicating the reduction of the microorganisms in longer periods in the presence of cold plasma irradiation. Furthermore, a study of milk proteins, which were quantitatively analyzed using SDS-PAGE, indicated that total milk proteins did not change significantly before and after plasma exposure (31). In another study, the effects of cold plasma were evaluated on the amount of *E. coli* bacteria in raw milk with different fat contents. In the

mentioned study, the cold plasma produced on semi-skimmed and nonfat milk at intervals of zero, three, six, nine, 12, 15, and 20 minutes was used, and a 54% reduction was reported in the general bacterial population after only three minutes regardless of the milk fat content; this is inconsistent with the results of the present study. In addition, no significant changes were observed in the color of the milk samples and the pH of raw milk in the mentioned research, and no viable cells were detected in the whole milk samples after one week. As a result, the samples remained without live cells during the six-week storage period. Based on these findings, it could be concluded that the applied technology could reduce the *E. coli* bacteria in milk by more than three times without significantly changing the pH or color of milk (32). This technology has also proven effective in eliminating other pathogens. For instance, a study assessed the effects of cold plasma on inactivating 20 strains of *Prototheca zopfii* extracted from cow's milk. Notably, this pathogen could cause mastitis in cows and appear in their milk. This microorganism is highly resistant to antibacterial and antifungal agents, as well as rising temperatures. The results of the mentioned study indicated that cold plasma could be an effective method against *P. zopfii* (33).

In another study, the function of plasma was evaluated in milk sterilization, and its effects on the structure and physicochemical properties of milk were also investigated. According to the obtained results, the produced plasma affected the bacterial cell membrane, metabolic enzymes, and DNA and decreased enzyme activity and DNA and membrane degradation, as well as the acceptable physicochemical properties of the samples (color, viscosity, pH, and acid titration) in the treatments with the voltage of 70 and 80 for 120 seconds. Therefore, it was concluded that the new method could be used for milk sterilization (34).

Another research evaluated the effects of plasma produced by DBD at voltages of 1.5, 3, and 5 kV for three minutes and the constant frequency of 500 Hz at room temperature on the microorganisms of diluted and non-diluted raw milk. At the voltage of 3 kV for approximately three minutes, no significant change was observed in the pH of raw milk, and all bacteria were eliminated (19). The results of the present study also showed that the voltage of 10 kV and frequency of 33 kHz, as well as the voltage of 20 kV and frequency of 28 kHz, caused a significant reduction (twice) in the colony counts of *S. aureus* and *E. coli*.

Conclusion

Since heat treatments may lead to the loss of beneficial vitamins and even the molecular taste and profile of milk, using new non-thermal methods, such as cold atmospheric plasma, could result in the production of foods with minimal pathogens, which could even have health benefits. Some of these benefits include the significant reduction of waste and environmental pollution, lower energy consumption compared to thermal methods, and the reduction of the microbial load in heat-resistant agents. It is hoped that by using non-thermal pasteurization methods such as cold atmospheric plasma, a new step would be taken toward the improvement of the Iranian food industry.

Conflicts of Interest

None declared.

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