Determination of the effect of milk fat on the inactivation of *Listeria monocytogenes* by ohmic heating

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ABSTRACT

Research in recent years has focused on innovative technologies that provide pathogen inactivation without damaging the structural properties of foods. Ohmic heating (OH) is an innovative technology, that provides an effective microbial inactivation with massive and rapid heating. This study aims to determine the effects of milk fat on the inactivation of *Listeria monocytogenes* by OH with a low voltage gradient. *L. monocytogenes* (ATCC 13932) inoculated 3.1%, 1.5%, and 0.1% fat-milk samples were heated up to 62°C by OH and conventional heating (CH) process. OH treatment lead to the inactivation of *L. monocytogenes* in both 1.5% and 0.1% groups and led to approximately 5.30 log decrease, however, there was a 3.10 log decrease in the 3.1% group at 6 min. CH lead to a few reduction as 0.21, 0.29 and 0.39 log in 3.1%, 1.5% and 0.1% fat-milk respectively. In OH, the sublethal injury ratio was higher than CH in all milk groups. However, OH did not statistically change color and pH values at the 6th min of the process, had a significant effect on hydroxymethylfurfural value only in 3.1% fat-milk. In conclusion, the increased fat content may have important inhibitory effects on pathogen inactivation in OH. Thus, the OH conditions should be chosen carefully to sufficient inactivation of pathogens in milk with high-fat content.

Introduction

Listeria monocytogenes is a harmful foodborne pathogen found in milk and dairy products, resulting in foodborne diseases and deaths (4). Due to its ubiquitous feature, *L. monocytogenes* can spread widely to the environment and maintain its vitality even under adverse conditions such as cooling, freezing, heating, and drying (26).

Inadequately heat-treated foods cause the majority of foodborne infections in many parts of the world. Several preservation methods, including thermal and non-thermal treatments, have been applied for many years to inactivate foodborne pathogens and maintain food safety (19). Thermal treatment, which has long been utilized to prevent foodborne infections, has a fundamental constraint in terms of quality degradation caused by severe heat. Research in recent years has focused on innovative technologies that provide pathogen inactivation without damaging the structural properties of foods. Ohmic heating (OH) is an innovative technology that provides an effective microbial inactivation with massive and rapid heating. In this technic, heat is generated when an alternative current passes directly through foodstuff (9, 10). OH has been gained much attention in the food industry due to its rapid, uniform heating distribution to provide food safety with minimal changes in structure, nutrition, or sensory attributes in foods (1, 2, 20, 21, 24).

Many researchers have recently researched the use of ohmic heating to inactivate foodborne bacteria (13, 18, 22). Previous studies showed that OH is affected by extrinsic factors such as voltage and frequency and intrinsic factors such as features of lipids, proteins, carbohydrates and their quantities in food. Both of them significantly affect the electrical conductivity and pathogen inactivation (12, 13, 18).

Although OH has been widely used in the processing of milk products, to the best of our knowledge, research

on the combined effect of milk fat and low voltage gradient on pathogen inactivation is quite limited. This study aimed to investigate the effect of milk fat and low voltage gradient on the inactivation of *L. monocytogenes* by OH and the determination of the quality changes.

Materials and Methods

Bacterial strain and cultural condition: American Type Culture Collection (ATCC) standard *L. monocytogenes* 4b (ATCC 13932) were used in this study. Stock cultures were transferred in Tryptic Soy Broth (BK046HA, BIOKAR) and incubated aerobically overnight at 37°C before experiments. Bacterial cultures were centrifuged at 5000 rpm for 5 min at 4°C, and the pellets were washed twice in 9 ml 0.9% saline with thorough mixing by a vortex. After centrifugation, the bacterial pellets were re-suspended in the same buffer. The bacterial suspensions were serially diluted in 0.1 % peptone water and plated on COMPAS Listeria agar (BK192HA, BIOKAR) with the spread plate technique for the enumeration. Plates were incubated for 24-48 h at 37°C before counting colonies. All bacterial suspensions were confirmed to be contained approximately 10^7 bacterial cells mL^{-1}.

Sample preparation and inoculation: A total of 72 UHT milk samples packed in 200 mL aseptic tetra pack containers were purchased from a local market in the province of Burdur, Türkiye. The whole (3.1%, n=24), semi-skimmed (1.5%, n=24), and skimmed (0.1%, n=24) UHT milk kept at +4 °C until the experiments were carried out. Before inoculation, milk samples were plated onto Nutrient Agar (105450, Merck) at 37 °C for 24-48 h, and there was no colony detected. The harvested pellet of *L. monocytogenes* strains was re-suspended in 9 mL 3.1%, 1.5%, and 0.1% UHT milk samples, respectively. The final concentration of *L. monocytogenes* cells in milk was confirmed by spreading the dilutions on plates and it was approximately 10^7 CFU/mL.

Experimental equipment: OH was carried out by a laboratory-type OH unit consisting of food stuff-grade stainless steel electrodes (304 L), a K-type thermocouple, a microprocessor (ERAK-TEK, Konya), a personal computer, a power supply (VARSAN, İstanbul) providing AC, 50Hz, 10 A and output voltage range 0-250 V, a magnetic stirrer and a heating unit. Time and temperature changes were recorded during the heating process through the microprocessor linked to the personal computer (Figure1). The heating unit consisted of a glass vessel of 300 mL capacity and two food stuff-grade stainless steel electrodes (electrode gap 5 cm). The K-type thermocouple was inserted into the unit's center to monitor temperature during the OH process.

Two hundred mL milk samples inoculated (0.05%,1 mL) with *L. monocytogenes* were subjected to 10 V/cm and 50 Hz in the OH treatment. All experiments were started from the initial state at 23.8 °C, and continued until the temperature at the center of the milk reached 62°C (it takes approximately 6 min and 20 min for OH and CH, respectively). This temperature was considered the end of the heating process. For the conventional heating treatment, a 200 mL sample was heated using a digital hot water bath (WB14, Memmert, Germany) (set temperature: 62 ºC) by the same heating procedure. During the heating process, 1 mL of milk sample, at the center of the heating unit was taken out, respectively, by one-off sterile injector at appropriate intervals (2 min), and immediately cooled in an ice-water bath.

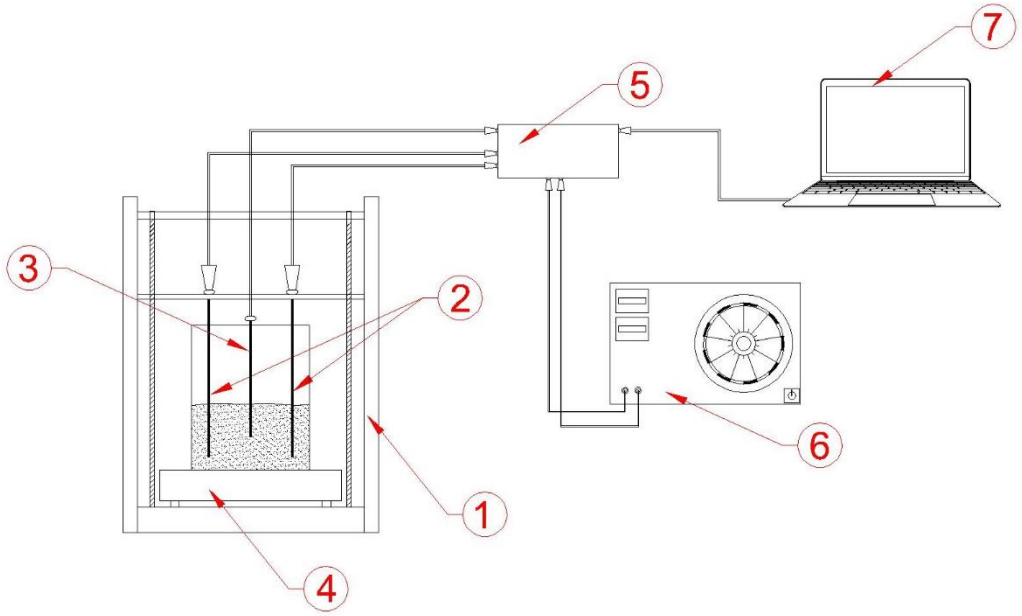

Figure 1. Schematic diagram of the OH system. 1: Ohmic cell, 2: Electrode, 3: Thermocouple sensor, 4: Magnetic stirrer, 5: Microprocessor, 6: AC power supply, 7: Computer.

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Enumeration of viable and injured cells: Spread plate method was used to enumerate the *L. monocytogenes* population after the heating treatment. One mL of cooled milk samples were serially diluted in 0.1% peptone water, and 0.1 mL samples were spread plated onto selective Compass Listeria Agar (CL) to count noninjured and were spread onto Compass Listeria Agar + TSA (CLTSA) to count both injured and noninjured bacterial cells. All plates were incubated for 24–48 h at 37°C before counting colonies. The sublethal ratio was calculated according to the following equation:

$$\text{The sublethal ratio (\%)} = 100 - [(\text{CFU/mL CL})/(\text{CFU/mL CLTSA})] \times 100$$

Where CFU/mL CL was the counts on Compass Listeria Agar; CFU/ mL CLTSA was the counts on Compass Listeria Agar +TSA (22).

Color, pH, and hydroxymethylfurfural value: The color of the samples was measured using a Konica Minolta CR400 colorimeter (Konica Minolta, Osaka, Japan). The hydroxymethylfurfural (HMF) content of the samples was determined according to the method described by Keeney and Bassette (11). The absorbance of the samples was calculated at 443 nm using a spectrophotometer (Thermo-MultiScan GO, ThermoScientific™). The standard curve was prepared using the HMF standard (Sigma, 1-10 µM/mL), and the results were expressed as µM/L. The pH was measured with a pH-meter (WTW Lab-pH Meter inoLab® pH 7110) at room temperature. Measurements were carried out in triplicate.

Statistical analysis: The mean values were obtained from triplicate trials. The results were analyzed by one-way analysis of variance (ANOVA) and T-test using SPSS software (Version 21.0; SPSS Inc., IBM Corporation, USA). Significant differences between the treatments were examined by Duncan's test and the results were expressed as mean ± standard deviation ($P<0.05$).

Results

The temperature changes of the samples inoculated with *L.monocytogenes* treated with OH and CH were presented in Figure 2. The initial temperature was 23.8-24.0 ºC ($P>0.05$). All OH-processed samples showed a similar linearly increasing trend in temperature. In all milk samples required approximately 6 min to reach 62 ºC. In the CH-treated samples, the rate of temperature increase dropped progressively, taking 20 min, to reach 62 ºC. Temperature rise was more rapid in OH treatment than CH.

Survival rates of *L. monocytogenes* cells during OH and CH in milk with different fat rates are shown in Table 1. In the OH experiment, the initial counts of *L. monocytogenes* cells were almost the same in all experimental milk samples. Also, the counts of *L. monocytogenes* remained stable ($P>0.05$) throughout the first 4 min of OH treatments while the temperature was below 50ºC (Figure 2). After the 4th min of the OH experiment, microbial counts of 1.5% and 0.1% fat-milk samples had already been reduced and the reductions of both experimental groups were greater than those in 3.1% groups ($P<0.05$). At the end of the 6th min, *L. monocytogenes* were inactivated in both 1.5% and 0.1% groups and led to approximately 5.30 log CFU/mL decrease ($P<0.05$). However, there was a 3.10 log CFU/mL decrease in the 3.1% group at the same process time ($P<0.05$).

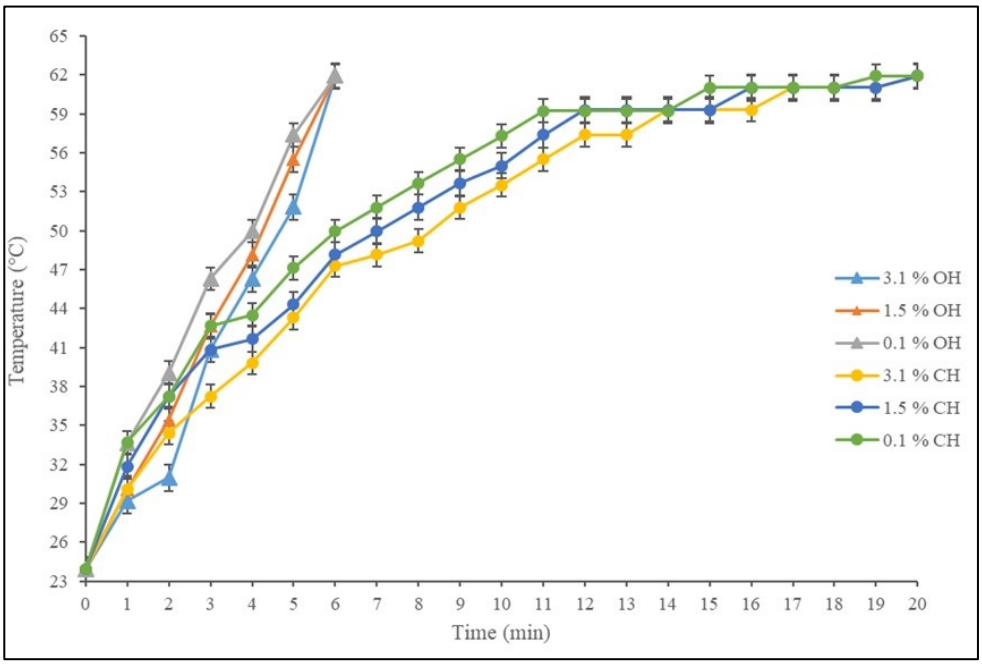

Figure 2. Time-temperature profiles of ohmic and conventional heating of milk samples with different fat contents. OH: Ohmic heating, CH: Conventional heating.

Table 1. Inactivation of *L.monocytogenes* cells by OH and CH in milk with different fat content at 10 V/cm voltage gradient.

Experiment	Time (min)	Colony counts (log CFU/mL)		
		3.1%	1.5%	0.1%
OH	**0**	5.32 ± 0.14^{x}	5.33 ± 0.03^{x}	5.32 ± 0.02^{x}
	2	5.35 ± 0.14^{x}	5.32 ± 0.01^{x}	5.32 ± 0.01^{x}
	4	5.33 ± 0.11^{x}	5.25 ± 0.05^{y}	5.22 ± 0.05^{yB}
	6	2.21 ± 0.28^{yaB}	ND^{zbB}	ND^{zbB}
CH	**0**	5.30 ± 0.12^{x}	5.30 ± 0.12^{x}	5.32 ± 0.07^{x}
	2	5.34 ± 0.08^{x}	5.34 ± 0.03^{x}	5.31 ± 0.03^{x}
	4	5.34 ± 0.18^{x}	5.31 ± 0.12^{x}	5.32 ± 0.02^{xA}
	6	5.09 ± 0.09^{yaA}	5.01 ± 0.10^{yabA}	4.93 ± 0.08^{ycA}

Values were means ± standard deviation of three replicates. ND: Not detected.
[x-z ,A-B] Values with different superscripts within columns differ significantly (P<0.05).
[a-c] Values with different superscripts within raws differ significantly (P<0.05).
OH: Ohmic heating, CH: Conventional heating.

In the CH experiment, the initial counts of *L. monocytogenes* cells were the same in all groups. During the CH, there was no change between the experimental groups at the 2nd and 4th min of experiments (P>0.05). At the end of the 6th min, there were a few reductions as 0.21, 0.29, and 0.39 log CFU/mL in 3.1%, 1.5%, and 0.1% groups, respectively (P<0.05), and the microbial reduction in 0.1% was higher than the others. Moreover, the reduction of *L. monocytogenes* microbial counts was significantly different (P<0.05) between the OH and CH treatments at each time point.

In this study, it was determined that the sublethal injury ratio increased with time (Figure 3). However, OH had a higher injury ratio than CH treatments at the same time. In this investigation, the maximum damage ratios of OH-treated *L. monocytogenes* in 3.1%, 1.5% and, 0.1% fat milk were 38.48%, 21.63%, and 20.50%, respectively, while the values for CH-treatments were 31.7%, 15.7%, and 17.14%, respectively. Due to the inactivation of *L. monocytogenes* at the 6th min of OH treatment in both 1.5% and 0.1% groups, the rate of sublethal injury could not be calculated.

Color values of milk samples were presented in Table 2. The initial *L** values were similar in 3.1% and 1.5% fat milk (90.74±0.89 and 89.29±2.05; P>0.05), while the initial *L** value of 0.1% fat milk (86.35 ±1.27; P<0.05) is statistically different. This difference observed in the initial color values could be due to the differences in fat content of the samples (3). However, generally, all OH-treated samples' *L**, *a** and *b** values did not significantly differ according to treatment times (P>0.05). CH-treated samples showed a significant increase in 3.1% and 0.1% groups in *L** and *a** values (P<0.05) according to treatment times, respectively, but not in *b** value (P> 0.05).

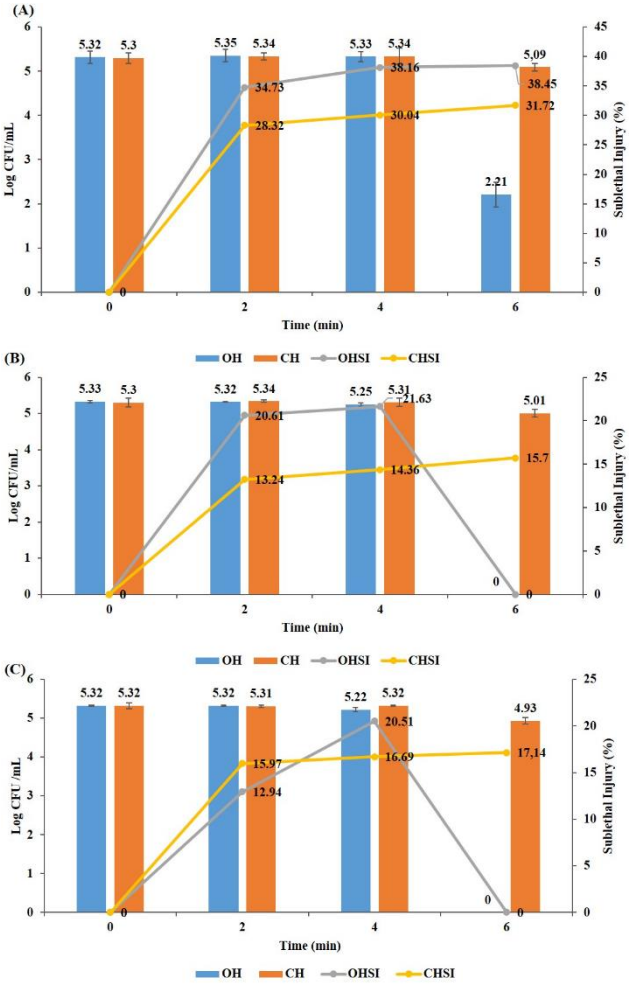

Figure 3. Inactivation of *L.monocytogenes* in milk samples by OH and CH. (A) 3.1% (B) 1.5%, (C) 0.1% fat milk. The columns represented the colony forming units, and the lines represented injury ratio (%). OH: Ohmic heating, CH: Conventional heating, OHSI: Ohmic heating sublethal injury ratio, CHSI: conventional heating sublethal injury ratio.

Table 2. Color values of milk samples.

		Milk Group (fat %)								
		3.1%			1.5%			0.1%		
	Time (min)	*L**	*a**	*b**	*L**	*a**	*b**	*L**	*a**	*b**
OH	0	90.74±0.89^{x}	-2.48±0.07^{x}	7.62±0.25^{y}	89.29±2.05^{x}	-3.52±0.23^{y}	7.33±2.26^{y}	86.35±1.27^{y}	-3.35±0.37^{y}	5.61±0.86^{y}
	2	92.73±0.21^{x}	-2.46±0.11^{x}	8.54±0.53^{x}	89.17±1.18^{y}	-3.50±0.39^{y}	6.58±1.99^{xy}	86.11±1.79^{z}	-3.54±0.42^{y}	5.18±1.58^{y}
	4	91.32±0.99^{x}	-2.40±0.11^{x}	7.84±1.02^{x}	89.38±1.38^{y}	-3.54±0.30^{y}	6.00±0.99^{xy}	85.63±1.03^{z}	-3.55±0.07^{y}	5.47±1,42^{y}
	6	91.39±0.67^{x}	-2.42±0.06^{x}	7.66±0.80^{x}	89.92±0.87^{x}	-3.43±0.26^{y}	6.78±1.53^{xy}	86.34±1.35^{y}	-3.50±0.15^{y}	5.53±1.33^{y}
CH	0	90.74±0.89^{bx}	-2.48±0.07^{x}	7.62±0.25	89.29±2.05^{x}	-3.52±0.23^{y}	7.33±2.26	86.35±1.27^{y}	-3.35±0.37^{by}	5.61±0.86
	2	92.53±0.64^{ax}	-2.53±0.04^{x}	8.09±1.35^{x}	90.69±0.94^{x}	-3.55±0.30^{y}	5.97±1.42^{y}	84.28±1.85^{y}	-3.71±0.09^{ay}	5.76±0.79^{y}
	4	93.05±0.26^{ax}	-2.60±0.02^{x}	8.55±1.22^{x}	90.81±0.85^{y}	-3.54±0.24^{y}	6.07±1.53^{y}	85.13±1.25^{z}	-3.70±0.07^{ay}	5.89±0.85^{y}
	6	92.67±0.43^{ax}	-2.52±0.03^{x}	8.14±1.23^{x}	90.56±0.84^{y}	-3.55±0.25^{y}	6.03±0.80^{y}	85.83±1.75^{z}	-3.70±0.13^{ay}	6.22±0.29^{y}

Values were means ± standard deviation of three replicates.
$^{x-z}$ Values with different superscripts within raws differ significantly (P<0.05).
$^{a-c}$ Values with different superscripts within columns differ significantly (P<0.05).
OH: Ohmic heating, CH: Conventional heating.

Table 3. HMF and pH values of the milk samples.

			Milk Group (fat %)		
		Time (min)	3.1%	1.5%	0.1%
HMF (µM/L)	OH	0	8.09±0.23^{ya}	6.34±0.32^{b}	7.72±0.54^{a}
		2	8.25±0.71^{y}	6.98±1.34	8.34±0.37
		4	8.30±0.30^{y}	7.06±1.53	8.99±2.00
		6	9.17±0.33^{xa}	7.11±0.71^{b}	9.06±0.66^{a}
	CH	0	7.95±0.97^{a}	6.34±0.32^{b}	7.72±0.54^{a}
		2	8.73±0.91^{a}	6.45±0.61^{b}	8.14±0.68^{a}
		4	9.96±1.27^{a}	7.06±1.53^{b}	8.19±1.09^{b}
		6	8.54±1.41	7.07±1.39	8.25±1.37
pH	OH	0	6.54±0.07^{b}	6.67±0.02^{a}	6.53±0.02^{b}
		2	6.61±0.16	6.56±0.05	6.57±0.02
		4	6.57±0.14	6.55±0.05	6.54±0.03
		6	6.59±0.20	6.52±0.11	6.55±0.02
	CH	0	6.54±0.07^{b}	6.52±0.10^{a}	6.47±0.08^{b}
		2	6.61±0.17	6.55±0.10	6.48±0.08
		4	6.57±0.18	6.49±0.12	6.47±0.11
		6	6.56±0.19	6.49±0.13	6.46±0.14

Values were means ± standard deviation of three replicates.
$^{x-z}$ Values with different superscripts within columns differ significantly (P<0.05).
$^{a-c}$ Values with different superscripts within raws differ significantly (P<0.05).
OH: Ohmic heating, CH: Conventional heating.

Hydroxymethylfurfural and pH values of the milk samples treated with OH and CH were presented in Table 3. There was a difference in the HMF content of all groups at the initial of the OH process. It was determined that the OH process had a significant effect on HMF values only in 3.1% fat milk, and the highest HMF value is reached at the 6th min of the OH process (9.17µM/L) compared to other times (P<0.05). However, there was no difference between the process times in 1.5% and 0.1% fat milk groups (P>0.05). There was no statistically significant difference in the CH process in processing times in all milk groups (P>0.05). However, a significant difference was determined between the groups at the initial 2nd and 4th min of the CH process (P<0.05).

Before the heating treatments, the initial pH values of the 3.1%, 1.5% and 0.1% fat-milk were determined as 6.54±0.07, 6.67±0.02 and 6.53±0.02, respectively (Table 3). Although the initial pH values were statistically different between the sample groups, there was no significant difference between the groups according to the processing time during both processes of OH and CH (P>0.05).

Discussion and Conclusion

The inhibitory effect of milk fat content on the inactivation of *L. monocytogenes* by OH with a low voltage gradient was evaluated in the present study. The counts of *L. monocytogenes* cells in milk groups remained relatively stable during the first 4 min when the temperature was below 50°C of both OH and CB treatments (Figure 2 and Figure 3), which could be attributed to the fact that 50°C treatment was regarded as the initial stage to cause protein denaturation and cell components (6). Therefore, CH treatment is insufficient to inactivate *L. monocytogenes* cells below 50°C considerably. After 6 min of the treatment (over 50°C), OH could inactivate bacterial cells in 1.5% and 0.1% fat-milk sample groups, probably due to the combined effect of both electroporation and heat generation during the OH treatment (8, 17, 18).

Electroporation occurred during the OH increases the cell permeability and may induce permanent cell damage by lead to leaking biological components such as amino acids, proteins, nucleic acids, and coenzymes. In this circumstance, the bacterial population may evolve into either a dead or sublethally injured subpopulation (23). In the present study, the percentage of sublethal injured and inactivated cells rose with increasing temperature in OH treated all milk groups. The sublethal damage ratios of CH-treated milk groups were lower than OH-treated samples. Similarly, Shao et al. (18) reported that OH showed a higher injury ratio in 6 min treatment compared to that of WB (approximately 60% and 30%, respectively).

In this study, reductions of *L. monocytogenes* counts were significantly different, corresponding to fat content and processing time. While fat content does not affect the inactivation of microorganisms during CH, the microbial inactivation with the OH process was affected by the properties of the food material processing conditions (temperature, frequency, and electric field strength). However, fat works as an insulator, and high-fat content is associated with lower electrical conductivity (13, 23).

The nutritional components of food, such as salts, sugars, proteins, and fats, increase bacteria's heat resistance (7). Espina et al. (7) reported that the nutritional components of apple juice had a protective effect on the thermal inactivation (54°C) of *E. coli* O157:H7. Kim et al. (13) also reported that both lactose and fat content had an inhibitory effect on the inactivation of *L. monocytogenes* by OH. Similarly, Kim and Kang (12) found that OH was more effective than CH at inactivating *L. monocytogenes* in skim milk and cream. The results of our study were also consistent with previous reports.

In the present study, color and pH values did not significantly change during the OH treatments. Also, there was no significant difference between OH and CH-treated milk groups. Similarly, Park et al. (16) found that the color and pH values of OH-treated samples did not differ from non-treated samples. Moreover, Shao et al. (18) reported that the color and pH values of OH-treated milk samples had no significant differences from that of CH treated and control samples.

Hydroxymethylfurfural is known as a marker of Maillard reaction (15). The HMF content of the samples remained stable during the CH treatment; however, initial heating of 4 min was stable and then increased slightly at 6 min in OH treatment. Initial HMF content of milk groups was significantly different from each other. The differences in HMF values observed from the initial heating processes may be due to brand differences in the milk used. In a study, the HMF content of whole, semi-skimmed, and skimmed milk from different brands were in the range of 4.90±0.27-12.74±0.63, 2.50±0.30-8.89±0.18, 2.16±0.37-7.49±0.11 µM/L, respectively (25). In another study, the total HMF content of commercial UHT and sterilized milk samples with different fat content was in the range of 3.46–5.75 µM/L and 15.52–21.38 µM/L, respectively (14). Morales and Jiménez-Pérez (14) concluded that the amount of fat and total HMF values were negatively correlated in UHT and sterilized milk, which was confirmed with our study results. Results achieved in the present study, the initial HMF content of all samples was much higher before the heating treatments. The result of the present investigation was in line with the above-reported work, probably because of the prolonged heating times of different commercial milk brands were used.

In conclusion, due to the non-thermal impacts, OH demonstrated a higher inactivation efficacy to CH treatment in a shorter heating time on *L. monocytogenes* in low-fat milk. Besides, there was no significant change between OH- and CH-treated samples regarding color and pH. These findings revealed that OH might efficiently inactivate *L.monocytogenes* with no significant difference in milk quality compared to CH treatment. However, the increased fat content protects bacteria from thermal damage in OH treatment. Thus, the OH treatment conditions should be chosen carefully to sufficient inactivation of pathogens in milk with high-fat content. These results may be useful in providing a reference for using OH, as a time and energy-saving thermal processing, in milk sterilization.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

Author Contributions

SÖ and HAK designed the experiments. SÖ carried out the analyses. HAK contributed to interpreting the results and took the lead in writing the manuscript. Both authors provided critical feedback and helped shape the research, analysis, and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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