Acta Scientiarum

http://periodicos.uem.br/ojs
ISSN on-line: 1807-8664
Doi: 10.4025/actascitechnol.v46i1.69714



# Inactivation of *Listeria monocytogenes* in ready-to-consume liquid infant milk treated with Ohmic Heating

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**ABSTRACT.** Ohmic heating is a promising alternative method to conventional heating for microbial inactivation. This study aimed to inactivate *Listeria monocytogenes* and assess some quality parameters in ready-to-consume liquid infant milk treated with different voltage gradients of ohmic heating. Different ohmic heating voltage gradients (5, 10, and 20V $cm^{-1}$) were applied to the samples inoculated with *Listeria monocytogenes* (ATCC 13932) for 5 minutes. The application of 20 V $cm^{-1}$ ohmic heating induced inactivation of *Listeria monocytogenes* at 4 minutes and resulted in approximately 5.34 log reduction; however, there was no significant reduction for the 5 and 10V $cm^{-1}$ groups. Moreover, 20V $cm^{-1}$ ohmic heating application did not cause any changes in pH, $L^*$, and $b^*$ values. A significant decrease in $a^*$ value and an increase in hydroxymethylfurfural value was noted in this group. In conclusion, the effectiveness of ohmic heating in pathogen inactivation depends on the applied electric field intensity and the application time. As a result, the ohmic heating conditions must be carefully determined for the infant milk to inactivate pathogens and ensure public health protection. The results of this study contain significant and beneficial data that can be disposed of the listeriosis risk associated with the consumption of infant milk in infants.

**Keywords:** Infant milk; *Listeria monocytogenes*; ohmic heating; foodborne pathogens.

Received on September 06, 2023.
Accepted on March 15, 2024.

## Introduction

Milk is an essential component of human nutrition; however, if hygienic rules are not followed during its production, processing, and storage, it can cause harmful effects on humans. Milk and dairy products may be responsible for a wide variety of foodborne illnesses because of the favorable conditions they provide for the growth of bacteria. Babies and children need a large amount of breast milk, infant formula for their early nutritional needs, and infant milk. The high protein, lipid, and glucose content of infant formula allows it to mimic the nutritional profile of breast milk, and it is available in powder, liquid concentrate, and ready-to-feed versions. The high concentration of its constituents is the primary factor in its susceptibility to deterioration by microorganisms (O'Connor, 2009).

A significant public health concern, *Listeria monocytogenes* is a highly adaptable bacterium that can survive in a broad range of temperatures and environmental conditions, including growth in the refrigerator (Buchanan, Gorris, Hayman, Jackson & Whiting, 2017). Milk represents one of the most important sources of transmission of *L. monocytogenes* to humans (Erol & Taşçi, 2021, Rugji & Dinçoğlu, 2022; Midam, Erol, Rugji, Sen, & Tasçi, 2023; Rugji et al., 2023). Most foodborne infections worldwide are associated with inadequately heat-treated foods. The main reason for preserving foods with heat treatment is to obtain microbiologically reliable products with the least loss of quality properties and nutritional value. The pasteurization process applied to milk is an effective technique that prolongs the shelf life of the product by neutralizing pathogenic microorganisms and enzymes in milk. However, the processing time applied for the inactivation of pathogenic microorganisms may cause some adverse effects on the aroma and nutritional properties of the product (Cho et al., 2017). Alternative processing methods that prevent all the adverse effects of heat treatment and ensure safe products are gaining importance today.

Ohmic heating (OH) is based on heating the food placed between two electrodes by its resistance to the applied electric current (Baysal, İçier, & Baysal, 2011). When the metal electrodes come into contact with the food, the electrons in the food move to the electrode of opposite polarity, causing heat generation. The OH is also an alternative method to thermal microbial inactivation mechanisms and provides effective microbial inactivation with massive and rapid heating (Park & Kang, 2013; Yildiz-Turp, Sengun, Kendirci, & Icier, 2013; Jaeger et al., 2016; Cho et al., 2017). The efficiency of the OH process is affected by internal (fat, protein, and carbohydrate content of the food) and external factors (voltage and frequency) (Baysal & Icier, 2010; Lee, Ryu, & Kang, 2013). The electrical conductivity of foods varies depending on the internal factors of the food and may have a protective effect on the inactivation of pathogens (Kim & Kang, 2015; Özkale & Kahraman, 2023; Ayyıldız & Kahraman, 2024). Although there are many publications on OH applied in different solid and liquid media (Park & Kang, 2013; Kim & Kang, 2015; Cho et al., 2017; Tian et al., 2019; Pires et al., 2020; Kahraman & Gacar, 2023; Özkale & Kahraman, 2023), to the best of our knowledge, research on the inactivation of *L. monocytogenes* with OH in ready-to-consume liquid infant milk is quite limited. This study aimed to investigate the inactivation level of *L. monocytogenes* in infant milk treated with different voltage gradients of OH and some changes in quality parameters.

## Material and Methods

### Sample preparation

In this study, 200 mL Ultra High Temperature (UHT) ready-to-consume liquid infant milk samples (n= 30, Milupa®, Turkey) were purchased from the local markets of Burdur province of Turkiye. Samples were stored in a refrigerator (+4 °C) until the experiments. Infant milk samples were spread onto Nutrient Agar (Sigma, Germany) before bacterial inoculation and incubated at 37 °C for 24-48 hours and no bacterial colony was detected. *L. monocytogenes* 4b (ATCC 13932) cells were cultured in Tryptic Soy Broth (Merck, Germany) for 24 h at 37 °C. The bacterial suspension was centrifuged at 4000 rpm for 5 min at 4 °C. The pellet was washed with sterile 0.9 % NaCl and re-suspended in the milk. To enumerate bacterial cells, they were serially diluted in 0.1% peptone water and sprouted growth on Oxford agar (Sigma, Germany). The plates were then incubated at 37°C for 24-48 hours. The final concentration of *L. monocytogenes* cells in milk was confirmed as approximately $10^7$ CFU $mL^{-1}$ by spreading plate technique.

### Experimental equipment

The OH unit used in the study is based on our previous study (Özkale & Kahraman, 2023) was used in this study. Briefly, the OH device consisted of 304 L stainless steel electrodes, a K-type thermocouple, a microprocessor, a personal computer, a power supply (AC, 50Hz, 10 A, 0-250 V), a magnetic stirrer, and a heating unit. Time and temperature changes were recorded during the heating process using a microprocessor linked to a personal computer. Two hundred mL milk samples inoculated with *L. monocytogenes* (1 mL) were subjected to 5V $cm^{-1}$, 10V $cm^{-1}$, and 20V $cm^{-1}$ electric field in the OH treatment. All experiments began at 20 °C and were continued until the temperature at the core of the sample reached 62.5 °C.

### Enumeration of viable cells

One mL of milk sample cooled with ice was serially diluted in 0.1% peptone water. The dilutions were plated onto Oxford Agar (OX) to count the viable ones and onto Oxford Agar + TSA (OX-TSA) to count both injured and uninjured bacterial cells. All plates were incubated at 37°C for 24-48 hours before counting the colonies. The sub-lethal rate (%) was calculated according to Tian et al. (2019).

### pH determination of the samples

The pH of the samples was measured before and after the ohmic experiment using a pH meter (Testo 205, Lenzkirch). The calibration was performed using standard buffers (pH 7.01 and 4.01) before the measurement.

### Hydroxymetil-furfurole analyses (HMF)

The evaluation was performed according to the method of Morales & Jimenez-Perez (2001) with several minor modifications. The first step involved digesting 2 mL of milk at 100°C for 1 hour with 1 mL of 0.3 mol equi $L^{-1}$ oxalic acid solution in securely stopped Pyrex containers. After a rapid cooling in ice, the mixtures

were slowly de-proteinised using of trichloroacetic acetic acid (1 mL) solution (40%, w/v) and centrifuged at 11 000×g for 12 min at 4°C. Then the sample was filtrated through a 0.45-µm acetate filter (13 mm, MSI Inc., Westboro, MA) and was ready for HPLC analysis. HPLC conditions and settings of the applied method are given in Table 1.

**Table 1.** HPLC conditions and settings.

| Conditions | Settings |
|---|---|
| Instrument | Agilent Technologies 1200 infinity |
| Detector | DAD 285/4 nm, REF;360/100 nm |
| Column | C18, Generix 5C, 5 µm, (25x4,6 mm) |
| Column temperature | 30°C |
| Mobile phase | A, Methanol (10%) B: $H_20$(90%) |
| Injection volume | 10 µL |
| Flow rate | 1.0 mL $min^{-1}$ |

### Statistical analysis

The experiments were performed in triplicate. The data was analyzed with SPSS software (Version 21.0; SPSS Inc., IBM Corporation, USA) using one-way ANOVA and the T-test. Duncan's multiple range test was used to determine significant differences ($p < 0.05$).

## Results and discussion

In the current study, *L. monocytogenes* (ATCC 13932) inoculation was applied to infant milk samples. Then, the change in *L. monocytogenes* cell viability was determined after OH treatment with different electric field strengths for 5 min. OH process was initiated at ≈20°C milk temperature and samples were taken each min with a sterile syringe for evaluation. During the 5 min process, an average temperature of 62.5°C was reached in the 3rd min in the group of 20V $cm^{-1}$ electric field, while 5V $cm^{-1}$ and 10V $cm^{-1}$ voltage groups reached in 5 min 23°C and 36°C, respectively (Figure 1).

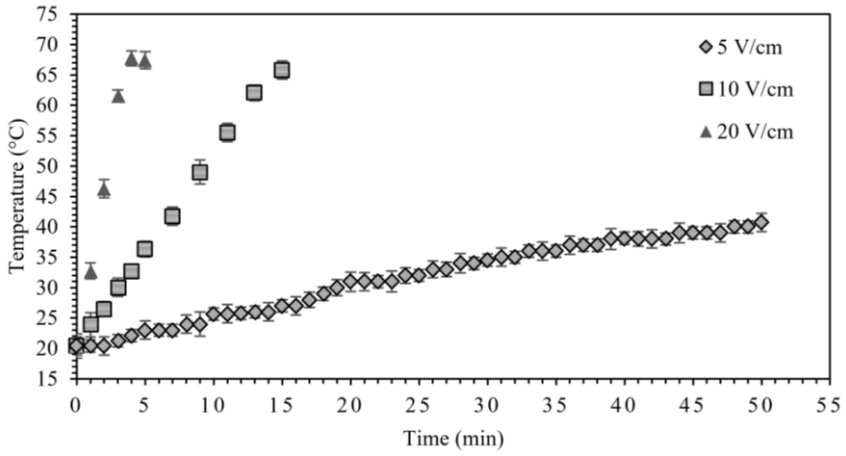


**Figure 1.** Time-temperature profiles of infant milk samples treated with 5V $cm^{-1}$, 10V $cm^{-1}$, and 20V $cm^{-1}$ OH.

The variations in the colony counts of *L. monocytogenes* after the OH treatment is given in Table 2. Different voltage gradients did not cause a significant difference in *L. monocytogenes* counts in the first 3 min ($p > 0.05$). *L. monocytogenes* counts at 4th and 5th min of OH treatment with voltage gradients of 5V $cm^{-1}$, 10V $cm^{-1}$, and 20V $cm^{-1}$ were 5.16±0.69, 4.31±0.14, and <1 log CFU $mL^{-1}$ and 5.12±0.10, 4.30±0.30, and <1 log CFU $mL^{-1}$, respectively, and the difference between the groups was significant ($p < 0.05$). The OH application voltage gradients of 5V $cm^{-1}$, 10V $cm^{-1}$, and 20V $cm^{-1}$ resulted in 0.18, 1.03, and 5.34 log CFU $mL^{-1}$ reductions, respectively, from baseline *L. monocytogenes* counts at the 4th min of treatment. It was observed that the decrease in *L. monocytogenes* numbers of 20V $cm^{-1}$ OH application was statistically significant from the 4th min compared to the beginning of the experiment ($p < 0.05$). 20V $cm^{-1}$ OH treatment completely inhibited all *L. monocytogenes* inoculated into milk samples from the 4th min.

**Table 2.** Change in *L. monocytogenes* counts after 5V $cm^{-1}$, 10V $cm^{-1}$, and 20V $cm^{-1}$ voltage gradient (log 10 CFU $mL^{-1}$, $p < 0.05$).

| Experiment time (min) | 5 V $cm^{-1}$ | 10 V $cm^{-1}$ | 20 V $cm^{-1}$ |
|---|---|---|---|
| 0 | 5.34±0.05 | 5.34±0.09 | $5.34±0.32^{x}$ |
| 1 | 5.33±0.07 | 4.40±2.15 | $5.13±0.31^{x}$ |
| 2 | 5.30±0.16 | 4.50±1.71 | $4.23±2.07^{x}$ |
| 3 | 5.24±0.37 | 5.17±0.56 | $4.95±0.04^{x}$ |
| 4 | $5.16±0.69^{a}$ | $4.31±0.14^{b}$ | $<1^{cy}$ |
| 5 | $5.12±0.10^{a}$ | $4.30±0.30^{b}$ | $<1^{cy}$ |

Values were means ± standard deviation of three replicates. (<1 log CFU $mL^{-1}$= below the detection limit), a-c Values with different superscripts within rows differ significantly ($p < 0.05$) x-y Values within a column with different letters are significantly different ($p < 0.05$).

The count of sublethal-damaged cells (%) during the OH process is given in Figure 2. It was determined that the counts of sublethal-damaged cells gradually increased significantly in all three groups due to the increase in the treatment time ($p < 0.05$). During the OH procedure, the groups that had the highest counts of damaged *L. monocytogenes* cells (%) were found to be 20V $cm^{-1}$ ($p < 0.05$). Due to the inactivation of *L. monocytogenes* at the 4th and 5th min of 20V $cm^{-1}$ OH treatment, the rate of sublethal injury could not be calculated.

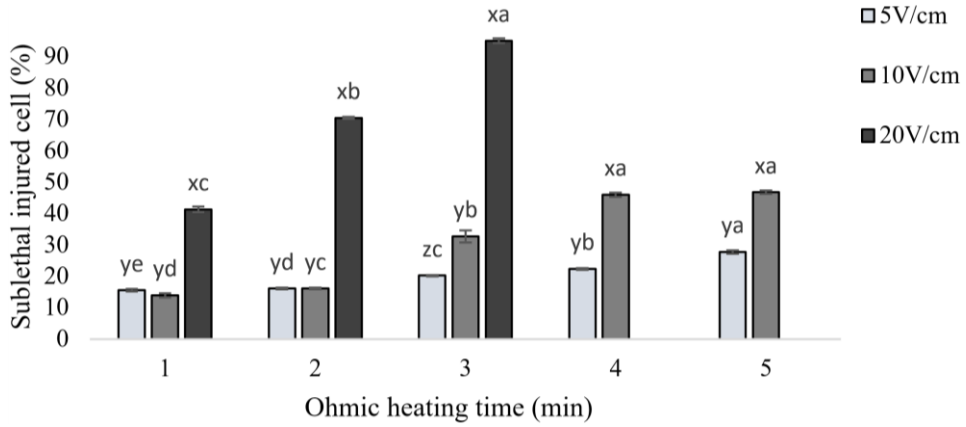


**Figure 2**. Sublethal injury levels (%) of *L. monocytogenes* in infant milk samples by 5V $cm^{-1}$, 10V $cm^{-1}$, and 20V $cm^{-1}$ OH treatments. Values with different superscripts in the same row (a-e) and in the same column (x-z) differ significantly ($p < 0.05$).

The pH and color values of milk samples are presented in Table 3. The initial pH values were similar and did not have any significant changes during the processing time according to treatment voltage gradients ($p > 0.05$). The OH treatments with 5, 10, and 20V $cm^{-1}$ voltage gradients did not cause significant effects on pH ($p > 0.05$). Similarly, $L^*$ and $b^*$ values of the samples were akin in all treatment voltage gradients during the processing time ($p > 0.05$). The OH treatments with 5, 10, and 20V $cm^{-1}$ voltage gradients did not have significant effects on $L^*$ and $b^*$values of the samples ($p > 0.05$). Generally, $a^*$ values were significantly decreased depending on the increase in OH-voltage gradients and exposure time ($p < 0.05$). The lowest $a^*$ values were detected in 20V $cm^{-1}$ OH group ($p < 0.05$).

Table 4 reveals the HMF values of the infant milk samples before and after 5 min of OH treatment. There was a significant difference in of 10V $cm^{-1}$ and 20V $cm^{-1}$ groups after 5 min compared to HMF content of the initial unprocessed samples (6.13 ng $\mu L^{-1}$, ($p < 0.05$). Generally, the OH process led to a significant increase in HMF values depending on the increase in OH-voltage gradients ($p < 0.05$). The highest HMF value was noted at 20V $cm^{-1}$ (7.39 ng $\mu L^{-1}$) compared to 5V $cm^{-1}$ and 10V $cm^{-1}$ (6.22 and 6.60 ng $\mu L^{-1}$, respectively).

In the present study, OH applied with 20V $cm^{-1}$ electric voltage gradient reduced *L. monocytogenes* below the detection limit at the 4th min, while 5V $cm^{-1}$ and 10V $cm^{-1}$ voltage gradients did not provide a significant reduction even at the end of the 5th min because the temperature was below 50°C of both 5V $cm^{-1}$ and 10V $cm^{-1}$ treatments (Figure 1). This could be explained by the fact that 50°C was regarded as the initial stage of protein denaturation and cell component degradation (Dill, Ghosh, & Schmit, 2011; Özkale & Kahraman, 2023). Therefore, 5V $cm^{-1}$ and 10V $cm^{-1}$ OH applications for 5 min are insufficient to inactivate *L. monocytogenes* cells below 50 °C considerably. Inactivation of pathogenic bacteria by the electric current lead to electroporation in the cell membrane. During the OH treatment, electroporation increases cell permeability and may cause cell damage by leaking biological components in the cell (Tian, Yu, Wu, & Dai, 2018) As a result of increased cell membrane permeability, the resistance of bacteria to heat decreases (Lebovka,

Shynkaryk, & Vorobiev, 2007; Park & Kang, 2013). In a study investigating the inactivation level of *Streptococcus thermophilus* in milk by OH, OH increased the permeability of the bacterial cell membrane and caused non-thermal damage on the *S. thermophilus* cell membrane (Sun et al., 2011). In the current study, 20V $cm^{-1}$ voltage gradient led to inactivation and caused up to 95% sublethal damaged cells in the 3rd min, probably due to the combined effect of both electroporation and heat generation during the OH application (Rivas et al., 2013; Gavahian, Chu, & Sastry, 2018; Shao et al., 2021). This result reveals the effectiveness of this chosen voltage gradient intensity compared to the other two groups.

**Table 3.** pH and color changes of the milk samples treated with 5V $cm^{-1}$, 10V $cm^{-1}$ and 20V $cm^{-1}$.

| | Experiment time (min) | 5V $cm^{-1}$ | 10V $cm^{-1}$ | 20V $cm^{-1}$ |
|---|---|---|---|---|
| pH | initial | 6.77±0.04 | 6.77±0.04 | 6.77±0.04 |
| | 1 | 6.83±0.11 | 6.85±0.08 | 6.85±0.11 |
| | 2 | 6.81±0.10 | 6.88±0.14 | 6.84±0.16 |
| | 3 | 6.85±0.11 | 6.84±0.13 | 6.88±0.08 |
| | 4 | 6.83±0.11 | 6.81±0.18 | 6.86±0.12 |
| | 5 | 6.82±0.12 | 6.84±0.11 | 6.82±0.09 |
| *L** | initial | 84.10±1.00 | 84.10±1.00 | 84.10±1.00 |
| | 1 | 84.52±0.40 | 83.47±1.73 | 83.00±2.04 |
| | 2 | 83.89±0.33 | 82.39±0.14 | 80.58±1.27 |
| | 3 | 84.13±0.62 | 80.68±0.30 | 83.03±1.55 |
| | 4 | 84.43±0.11 | 82.14±2.04 | 81.94±1.32 |
| | 5 | 84.24±0.07 | 82.16±2.11 | 83.11±0.56 |
| *a** | initial | $2.70±0.07^{z}$ | $2.70±0.07^{yz}$ | $2.70±0.07^{xy}$ |
| | 1 | $2.71±0.01^{za}$ | $2.66±0.05^{yzb}$ | $2.64±0.03^{yb}$ |
| | 2 | $2.84±0.02^{ya}$ | $2.61±0.02^{zb}$ | $2.50±0.01^{zc}$ |
| | 3 | $2.90±0.03^{xya}$ | $2.76±0.04^{xyzb}$ | $2.60±0.04^{yzc}$ |
| | 4 | $3.01±0.06^{xa}$ | $2.80±0.12^{xyab}$ | $2.62±0.06^{yzc}$ |
| | 5 | $3.00±0.05^{xa}$ | $2.91±0.08^{xa}$ | $2.71±0.06^{xyb}$ |
| *b** | initial | 4.75±0.23 | 4.75±0.23 | 4.75±0.23 |
| | 1 | 4.72±0.01 | 4.05±0.29 | 4.24±0.06 |
| | 2 | 4.10±0.02 | 4.25±0.36 | 4.56±0.28 |
| | 3 | 4.21±0.18 | 4.20±0.28 | 4.19±0.04 |
| | 4 | $4.43±0.23^{a}$ | 4.30±0.09 | 4.46±0.07 |
| | 5 | $4.58±0.25^{a}$ | 4.45±0.04 | 4.43±0.1 |

Values were means ± standard deviation of three replicates. a-c Values with different superscripts within rows differ significantly ($p < 0.05$). x-z Values within a column with different letters are significantly different ($p < 0.05$).

**Table 4.** The results HMF values of samples treated with 5V $cm^{-1}$, 10V $cm^{-1}$ and 20V $cm^{-1}$ voltage gradient.

| | HMF ng $\mu L^{-1}$ | | |
|---|---|---|---|
| | 5V $cm^{-1}$ | 10V $cm^{-1}$ | 20V $cm^{-1}$ |
| initial | 6.13±0.04 | $6.13±0.04^{y}$ | $6.13±0.04^{y}$ |
| after 5 min | $6.22±0.02^{c}$ | $6.60±0.01^{xb}$ | $7.39±0.01^{xa}$ |

Values were means ± standard deviation of three replicates. a-c Values with different superscripts within rows differ significantly ($p < 0.05$). x-y Values within a column with different letters are significantly different ($p < 0.05$).

There is limited research on the OH process with different voltage gradients. Sagong et al. (2011) investigated the inactivation level of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *L. monocytogenes* by OH with 10-20V $cm^{-1}$ electric field strength in orange and tomato juice. The *L. monocytogenes* population decreased to 3.76 log CFU $mL^{-1}$ after the application of 15V $cm^{-1}$ voltage gradient for 180 s on orange juice and *L. monocytogenes* decreased below the detection limit (<1 log) after the 210 s application of 15V $cm^{-1}$ voltage gradient. They stated that, in order to inactivate all three pathogens in tomato juice, 20V $cm^{-1}$ voltage gradient should be applied for 90 s, while 15V $cm^{-1}$ should be applied for 180 s and 10V $cm^{-1}$ for 420 s. These results show that OH is beneficial for the inactivation of *E. coli* O157:H7, *S*. Typhimurium, and *L. monocytogenes*, and the effect of inactivation depends on the applied electric field strength, duration of application, pathogen species, and the type of food. Pereira et al. (2020) evaluated the inactivation kinetics of *L. monocytogenes* in whey dairy beverage processed with 6V $cm^{-1}$ electrical field and determined that OH treatment at 62.5 °C resulted in 2.10 log CFU $mL^{-1}$ $min^{-1}$ reduction rate of *L. monocytogenes*. Our results confirm that the effectiveness of OH in pathogen inactivation depends on the applied electric field intensity and the application time, and is in line with the results of Sagong et al. (2011) and Pereira et al. (2020).

In the current study, pH, $L^*$ (whiteness), and $b^*$ (yellowness) values did not change in all OH treatment groups during the processing time. However, higher HMF content and lower redness ($a^*$) values were observed depending on an increase in voltage gradient and processing time. Parmar, Singh, Meena, Borad, & Raju (2018) determined the changes in the color of milk being subjected to OH treatment. Results revealed that yellowness increased significantly ($p < 0.05$) with time owing to the formation of primary Maillard reaction products. However, the pH values of the treated milk samples decreased significantly. The white color of milk is primarily due to casein micelles found in milk. Heat-induced protein denaturation and aggregation to casein micelles, dephosphorylation of casein, breakdown of k-casein, and precipitation of soluble calcium phosphate on casein micelles all result in a decrease in milk whiteness (Parmar, Singh, Meena, Borad, & Raju, 2018). Rocha et al. (2022) highlighted that there were no unfavorable shifts in temperature, rheology, or color in high-protein vanilla-flavored milk treated with OH. Kim & Kang (2017) determined that OH resulted in a minor drop in milk pH values, while no changes were observed in terms of color evaluation.

HMF content is considered as a marker for the Maillard reaction (Morales & Jimenez-Perez, 2001). The initial HMF content of UHT milk groups has a significant impact on HMF content after the OH process. A study, conducted on whole, semi-skimmed, and skimmed milk showed that the HMF content was in the range of 315–1606 ng $\mu L^{-1}$ (Urgu, Saatli, Türk, & Koca, 2017). Morales & Jiménez-Pérez (2001) also showed that the total HMF content of commercial UHT milk samples with different fat content ranged from 436-725 ng $\mu L^{-1}$. In the presented study, the results of the HMF content achieved after the OH treatment were not as high as mentioned above-reported studies.

## Conclusion

*L. monocytogenes* is an important burden for the dairy industry. Alternative technologies are needed to provide food safety in the dairy chain with a minimum negative effect on the nutritional values of dairy products. Ohmic treatment has demonstrated its viability as an alternative technology for the inactivation of *L. monocytogenes*. In the current study, 20V $cm^{-1}$ electric field intensity reduced *L. monocytogenes* below the detection limit at the $4^{th}$ min, while 5V $cm^{-1}$ and 10V $cm^{-1}$ voltage gradients did not provide a significant reduction even at the end of the $5^{th}$ minute. Therefore, it reveals the effectiveness of this chosen voltage gradient intensity compared to the other two groups. The results of this study provided significant and beneficial data in terms of the use of OH to prevent the listeriosis risk associated with the consumption of infant milk in children.

## Acknowledgment

This study was supported by TUBİTAK 2209A Student Scientific Research Grant (grant no. 1919B012101093).

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