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Effects of Microwave Radiation, Organic Acid, and Salt Combination on the Survival of *Pseudomonas Aeruginosa* Inoculated onto Veal Meat in Refrigerated Storage

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ABSTRACT

Introduction:Various species of *Pseudomonas* are abundantly found in the environment. Although they are weak pathogens, these bacteria play a key role in food hygiene and human health as they are psychrotrophic bacteria and could grow and proliferate at refrigerated temperatures, producing proteolytic and lipolytic enzymes. The present study aimed to evaluate the effects of microwave radiation, organic acid, and salt combination on the survival of *Pseudomonas aeruginosa* inoculated in veal meat in refrigerated storage.

Methods: The simultaneous effects of various doses of microwave radiation and various concentrations of salt and lactic acid on *P.aeruginosa* inoculated onto veal meat were assessed. In total, 108 samples were evaluated in nine treatments on days zero, three, six, nine, 12, and 15 during chilled storage at the temperature of -4°C. The control group was treated with distilled water. The bacterial count was determined in Pseudomonas Agar medium, which indicated that various concentrations of acid and salt, as well as different microwave exposure times and their interactions, had significant effects on the mean logarithm number of bacteria during the refrigeration period. Data analysis was performed in SPSS version 21 using the mixed repeated measures ANOVA.

Results: The interactions between microwave radiation, organic acid, and salt caused further reduction in the bacteria count compared to the control group. A significant difference was observed in the bacterial count in the acid and salt treatments with the control group (P<0.001). The effects of the salt concentration and microwave duration were controlled using 2.5% and 5% lactic acid, which reduce the bacterial logarithm during the refrigeration period compared to the acid-free samples (P<0.001). In addition, 5% acid significantly decreased the bacterial count logarithm during the refrigeration period compared to the samples treated with 2.5% acid (P<0.001). By controlling the effects of acid concentration and microwave duration, use of 4% and 6% salt significantly decreased the bacterial count logarithm during the refrigeration period compared to the non-salt samples (P<0.001). On the other hand, the samples containing 6% and 4% salt had no significant difference in terms of the bacterial count logarithm during the refrigeration period (P=0.89). The microwave duration of nine seconds (65°C) could significantly decrease the bacterial logarithm compared to seven (55°C), five (45°C), and three seconds (30°C) (P<0.001). Moreover, use of lactic acid (5%) and salt (6%) caused the microwave duration to reduce from nine seconds (65°C) to five seconds (45°C) to complete the elimination of bacteria.

Conclusion: According to the results, use of organic acid, salt, and microwave irradiation for *P. aeruginosa* inoculated into meat during refrigerated storage reduced the bacterial count. Furthermore, the combination of microwave radiation, organic acid, and salt concentration decreased the bacterial count more significantly compared to the control group, and a significant difference was observed between the bacterial counts when each factor was used compared to the control group.

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Introduction

Pseudomonas aeruginosa is a gram-negative, rod-shaped bacterium that may cause various diseases in humans (1). *P. aeruginosa* is a multidrug-resistant pathogen with advanced antibiotic resistance mechanisms, as well as the major cause of hospital-acquired infections, ventilator-associated pneumonia, and various sepsis syndromes (1, 2). This organism is an opportunistic bacterium that could give rise to

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severe infections in the course of some diseases, most notably in cystic fibrosis and traumatic burns (2, 3). In general, *P. aeruginosa* affects immunocompromised patients (4, 5). Treatment of the infections caused by *P. aeruginosa* might be challenging due to its natural resistance to antibiotics (5, 6).

The most prominent feature of *P. aeruginosa* is its effect on foods, as well as its proteolytic and lipolytic properties (7, 8). These bacteria are aerobic and could rapidly grow in high-protein and fatty foods, such as meat. This phenomenon causes the production of slimes in food, which eventually leads to food spoilage (8, 9). Some chemical preservatives are used to prevent the growth of microorganisms and delay the degradation of foodstuffs, which has recently attracted the attention of researchers (10, 11). Some chemical preservatives have antimicrobial activity *in-vitro*, while their use as food additives may inhibit their desired properties (12, 13). As such, attempts have been made to investigate and develop efficient food preservation methods (14, 15).

Weak organic acids in preservative groups are used as additives in the food industry (15, 16). Organic acids affect pH homeostasis, which is essential to the control of adenosine triphosphate, RNA, protein synthesis, and DNA replication and growth, disrupting the transfer of materials (15-17). These acids include propionic acid, benzoic acid, sorbic acid, acetic acid, lactic acid, citric acid, and salicylic acid (16).

Sodium chloride (NaCl) has historically been used as a food preservative (18). This application is based on the fact that at high concentrations, salt exerts dehydration effects on various microorganisms (19, 20). At the concentration of 5%, salt solution causes plasmolyse (cellular shrinkage) and rupture of the microorganism (20, 21), which could be observed when high salt concentrations are added to fresh meat. In such case, both microbial cells and meat cells develop wrinkles, which lead to water loss and destruction of microbial cells or prevention of their growth (20, 21).

Adequate salt must be used in order to influence hypertonic conditions; higher concentrations of salt enhance its preservative and recovery effects (22). Moreover, microwave radiation could lead to the inhibition of microorganism growth by heating (23-25). There has been gradual progress in the industrial application of microwaves in food production processes (24, 26). Microwaves are the energy waves that primarily affect water and other polar molecules, causing these molecules to vibrate and build up thermal energy.

The present study aimed to assess the simultaneous effects of various concentrations of microwave radiation, lactic acid, and salt on *P. aeruginosa* inoculated into veal parts. In total, 108 specimens were evaluated in nine treatments on days zero, three, six, nine, 12, and 15. After refrigeration, microbial tests were performed, including *P. aeruginosa* count. The control group was treated with distilled water, and the bacterial count was carried out in Pseudomonas Agar medium. Furthermore, the effects of various concentrations of acid and salt and different microwave times on the bacterial count logarithm were assessed during 15 days of refrigeration using mixed repeated measures ANOVA. According to the findings, various concentrations of acid and salt, as well as different microwave times and their interactions, had significant effects on the mean bacterial count logarithm during the refrigeration period.

Materials and Methods

Main Protocol

The survival potency of *P. aeruginosa* was investigated based on various treatments with organic acid (2.5% and 5%), salt (4% and 6%), microwave radiation (zero, three, five, seven, and nine seconds), and the treatments were assessed during the mentioned storage times (days zero, three, six, nine, 12, and 15).

Experimental Procedures

In total, 108 specimens (meat/veal sections) were divided into nine groups, as follows:

1. Four times of microwave exposure using 2.5% lactic acid;
2. Four times of microwave exposure using 5% lactic acid;
3. Four times of microwave exposure using 4% salt (NaCl);
4. Four times of microwave exposure using 6% salt (NaCl);
5. Four times of microwave exposure using 2.5% lactic acid and 4% salt (NaCl);
6. Four times of microwave exposure using 2.5% lactic acid and 6% salt (NaCl);
7. Four times of microwave exposure using 5% lactic acid and 4% salt (NaCl);
8. Four times of microwave exposure using 5% lactic acid and 6% salt (NaCl);

9. Four times of microwave exposure using distilled water as control

Preparation for Inoculation

P. aeruginosa ATCC 9027 was used as the reference bacterial strain in the present study. To prepare the desired suspension, the reference strain was cultured on the brain heart infusion (BHI) agar. The culture plate was incubated at the temperature of 37°C for 24 hours. The bacterial suspension was prepared in sterile distilled water, the turbidity of which was adjusted to 0.5 McFarland using a spectrophotometer (wave length: 600 nm, absorbance: 0.08-0.1). The suspension contained $1.5*10^8$ bacteria/ml. For the inoculation, the bacterial suspension was adjusted to 106 bacteria/ml.

Acid Preparation

Organic acid (2.5% lactic acid) was prepared and sterilized using a sterile 45-micrometer filter cartridge.

Salt Preparation

Various salt concentrations (4% and 6%) were prepared and sterilized in an autoclave.

Microwave Radiation

A Samsung microwave apparatus operating at 900 Watts was used for microwave radiation. After each microwave radiation, the surface temperature of the meat samples was measured.

Preparation of the Veal Sections

Veal sections were purchased and transferred to the laboratory near ice. In the laboratory, each meat section was thoroughly washed with distilled water.

Disinfection of the Meat Sections

The meat sections were disinfected by immersion in 3% nanosil solution for 30 minutes in order to minimize the microorganisms on the surface of the sample. Afterwards, the sterilized, disinfected meat sections were washed thoroughly with distilled water three times.

Bacterial Inoculation

The meat sections were transferred to the prepared bacterial suspension. After 30 minutes at the temperature of 23°C, the meat sections were removed and placed in sterile plates to allow bacteria to adhere to their surface.

Acid Spray

After inoculation, the designated meat sections were sprayed with the desired organic acid (2.5% and 5%), and distilled water was used to spray the samples in the control group.

Salt Spray

Sterile salt water was sprayed on meat pieces with specific concentrations (4 and 6%) and placed in a sterile plate for 10 min.

Refrigerated Storage

The veal sections that were treated with acid, salt, and microwave radiation were transferred to a refrigerator in sterile plastic zip packs to be examined on days zero, three, six, nine, 12, and 15 after inoculation. On these days, 10 grams of the meat samples were mixed with 90 milliliters of distilled water using a bag mixer, and serial dilutions were prepared and cultured on the Pseudomonas Agar. After 24 hours of incubation at the temperature of 37°C, *Pseudomonas* colonies were enumerated using a colony counter.

Statistical Analyses

Data analysis was performed in SPSS version 21. The effects of various concentrations of organic acid, salt, and microwave time on the bacterial count logarithm during 15 days of refrigeration were evaluated using the mixed repeated measures ANOVA. The differences in the logarithmic variations of the bacterial counts were also assessed between the groups of acid, salt, and microwave radiation ($P<0.01$), and various concentrations of acid, salt, and microwave exposure and their combination had significant effects on the mean bacterial count logarithm during the refrigerated storage ($P<0.001$).

Results

Tables 1-3 show the mean, minimum, and maximum bacterial count logarithms in 36 treatments. As can be seen, various concentrations of organic acid, salt, microwave radiation, and their combinations had significant effects on the mean bacterial count logarithm during the refrigeration period.

By controlling the effects of the salt concentration and microwave radiation time, 2.5% and 5% lactic acid significantly reduced the bacterial count logarithm during the refrigeration period compared to the acidic samples ($P<0.001$). Furthermore, 5% lactic acid significantly decreased the bacterial count

logarithm during the refrigeration period as opposed to the treatment with 2.5% lactic acid (P<0.001).

By controlling the effects of acid concentration and microwave radiation time, 4% and 6% salt could significantly reduce the bacterial count logarithm during the refrigeration period compared to the non-salt treated samples (P<0.001). However, 6% and 4% salt had no significant difference in the bacterial count logarithm during the refrigerated storage (P=0.89).

Table-1-3: Average, standard deviation, minimum and maximum logarithm of bacterial count in groups treated with lactic acid (p<0.001)

acid concentration	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
.00	2.074	.034	2.007	2.142
2.50	1.056	.034	.988	1.124
5.00	.689	.034	.621	.758

Table-2-3: Average, standard deviation, minimum and maximum logarithm of bacterial count in groups treated with salt (p=0.89)

salt concentration	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
.00	1.698	.034	1.630	1.766
4.00	1.072	.034	1.004	1.139
6.00	1.050	.034	.982	1.117

Table 3-3: Average, standard deviation, minimum and maximum logarithm of bacterial count in groups treated with microwave radiation (p<0.001)

Microwave time	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
3.00	2.190	.039	2.111	2.268
5.00	1.711	.039	1.632	1.789
7.00	.895	.039	.816	.973
9.00	.298	.040	.218	.377

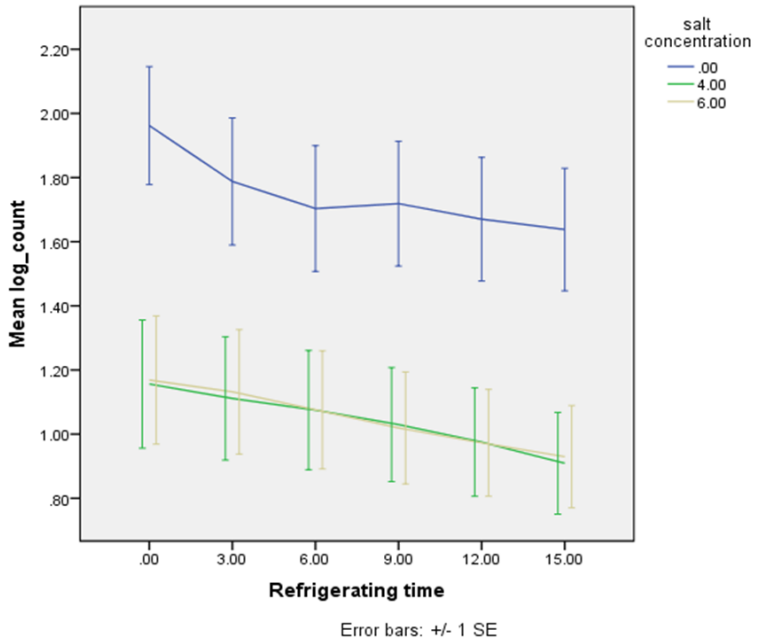

Figure-1-3: Effect of organic acids on Pseudomonas aeruginosa count inoculated on veal meat samples

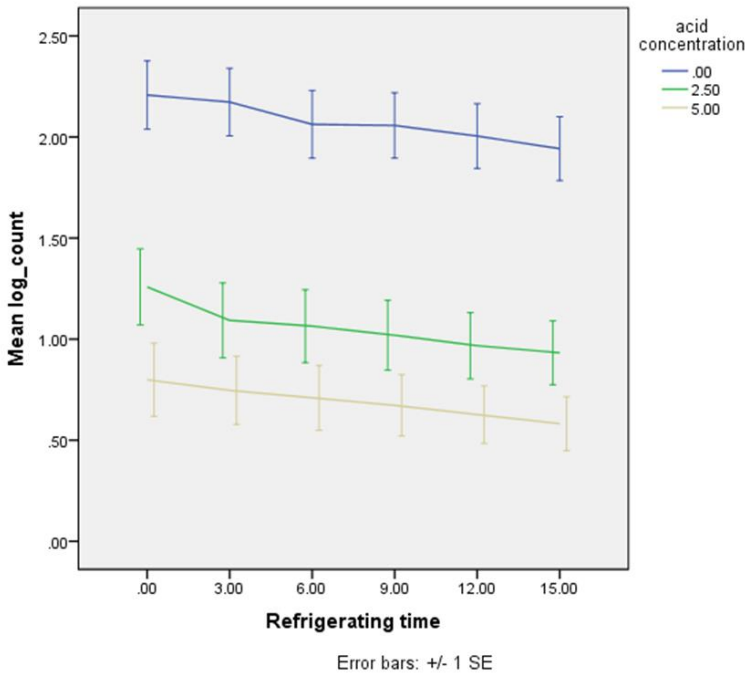

Figure 2-3: Effects of salt on Pseudomonas aeruginosa count inoculated on veal meat samples

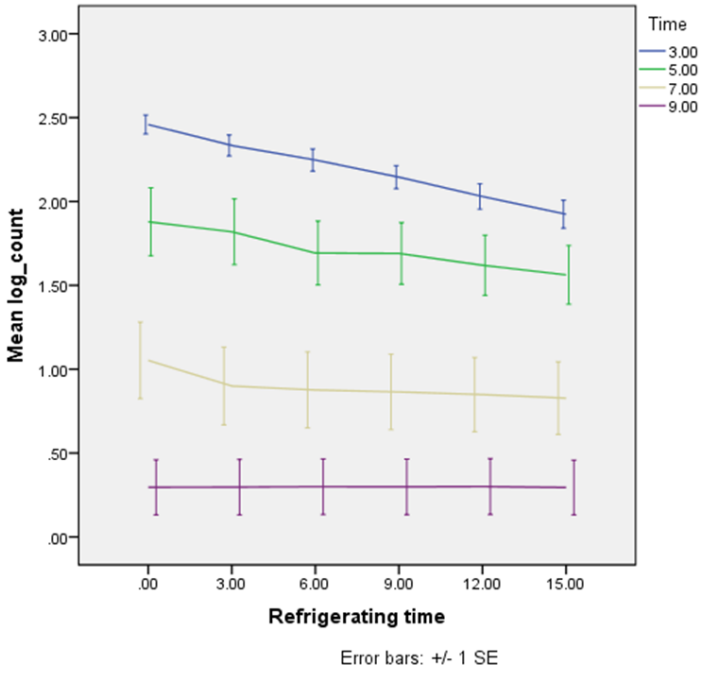

Figure -3-3: Effects of the microwave radiation on pseudomonas aeruginosa count inoculated on veal meat samples

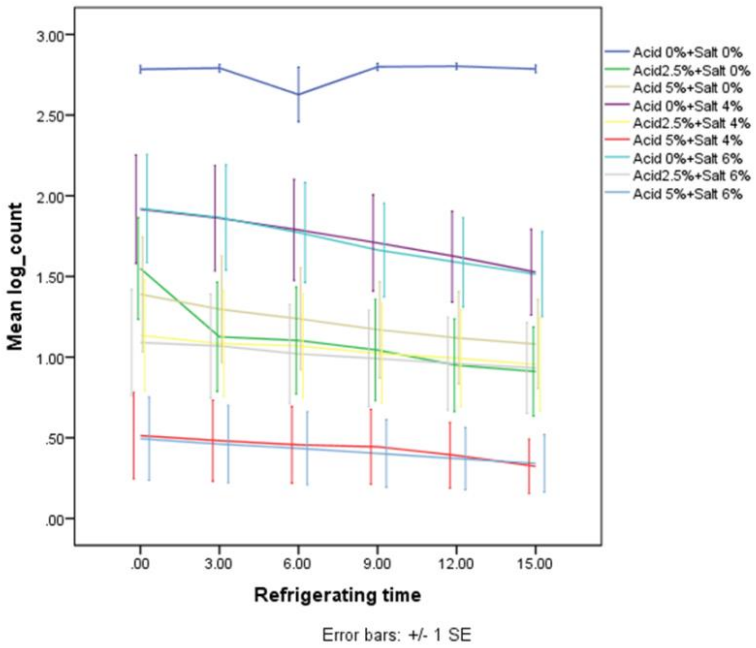

Figure -4-3: Effect of combination of salt, organic acid and microwave radiation on pseudomonas aeruginosa count inoculated on veal meat samples

By controlling the effects of acid and salt concentrations, the microwave radiation time of five, seven, and nine seconds significantly reduced the bacterial count logarithm during the refrigerated storage compared to the samples with the microwave duration of three seconds (P<0.001). Moreover, the microwave radiation time of seven and nine seconds significantly reduced the bacterial count logarithm during the refrigerated storage compared to the samples with the microwave radiation time of five seconds (P<0.001). Finally, the microwave radiation time of nine seconds significantly decreased the bacterial count logarithm during the refrigeration period compared to the samples with the microwave duration of seven seconds (P<0.001).

Time 3 s=30°C

Time 5 s=45°C

Time is 7 s=55°C

Time 9 s=65°C

Discussion

Health and safety of meat is an important challenge in food hygiene, which must be comprehended thoroughly. Effective management programs in this regard could only be achieved with the cooperation of all institutions, including the manufacturing, processing, distributing, supplying, and packing units (27, 28). Considering its biological properties and high nutrient levels, fresh meat is a food product with a high potential for rapid corruption. Several internal factors affect the protection period and freshening time of meat, such as the storage temperature, oxygen, internal enzymes, moisture, light, and microorganisms, which are most influential in this regard.

Considering their cold compatibility, *P. aeruginosa* species could grow at freezing temperatures and even at temperatures below zero, while they are normally destroyed during pasteurization (27, 28, 31). Another important feature of *P. aeruginosa* in terms of food products is their proteolytic and lipolytic properties, which cause undesirable odor and taste in food products through protein and fat degradation. These bacterial species are aerobic and grow rapidly on food products, making a surface to be licked due to slime production (30-32).

Numerous environmental factors affect the growth rate of microorganisms in food, such as temperature, pH, water activity (AW), atmosphere, and presence/absence of additives. It is has been confirmed that the combination of inhibitors and their interactions exert more potent effects on inhibiting the growth of microorganisms in food products compared to each factor separately (32-34). Moreover, several antibacterial reagents have been

suggested to destroy or decrease the number of pathogenic bacteria in meat products after slaughtering, including heating, washing with oxygen reagents (e.g., hypochlorite and peroxide), applying bacteriocins, organic acids (e.g., lactic acid, citric acid, and acetic acid), humectant compounds (e.g., salts and carbohydrates), and herbal additives (11).

Organic acids are the preservative agents that are extensively used in the food industry (15, 16); such examples are propionic acid, benzoic acid, sorbic acid, acetic acid, lactic acid, citric acid, and salicylic acid, which affect pH homeostasis. Proteins are responsible for the transportation of nutrients, and pH affects the protein activity in the cell membrane. Induced lowering of the pH denatures proteins, rendering them ineffective (35-38). Organic acids such as lactic acid, acetic acid, and citric acid that are used in food products are classified as GRAS preservatives, and their use in food has been reported to cause no adverse effects (40, 41).

Use of organic acids is an effective approach to the control or reduction of pathogenic and corrosive bacteria (e.g., *Salmonella* and *Pseudomonas*). *Pseudomonas* species play a key role in the corruption of the food products containing high levels of proteins and fat. The lethal effects of weak acids depend on the concentration (acid amount), acidity (pH), acid dissociation constant (Ka), and presence of other agents (e.g., salts or other factors) (42). Weak acids are common protectors that are effective at low concentrations and could reduce the growth rate of pathogenic bacteria through the proper reduction of pH (43).

Sodium chloride has historically been applied for food storage. The primary applications of salt include the storage of various meat products and elimination of corrosive and pathogenic agents. In this regard, high salinity levels have been shown to decrease the AW in food products and exert dehydration effects on various microorganisms (44). Addition of high concentrations of salt to fresh meat leads to the plasmolysis (shrinkage) of bacteria and their destruction. Such effect is due to the loss of water in meat and plasmolysis of the microbial infected cell, through which the microbe is damaged or inhibited in terms of growth. To this end, sufficient salt concentrations should be applied as higher salt concentrations enhance its maintenance effects.

The inhibitory effects of salt are independent of pH. With the salt concentrations of ≤20%, the growth of most bacteria could be prevented, while molds and halotolerant bacteria may grow even at high concentrations of salt (44). Controlling cold chains along with the use of growth inhibitors has proven effective in the growth inhibition of psychrophiles and other corruption agents, such as *Pseudomonas* species (45).

To date, several studies have investigated the effects of organic acids, microwave radiation, salt and their interactions on various bacteria during refrigeration. In the study by Arnatt et al. (1998), various species of *Pseudomonas* were assessed in the chicken meat stored in refrigeration units (3±0.5) on day zero, three, eight non-fluorescent species of Pseudomonas lansidins were identified. This species was sampled on day eight of corruption. (46).

In a research conducted by Ghazvini et al. (2011), the effects of three disinfectants of Nanosil and hydrogen peroxide were investigated, and Nanosil was reported to be stronger compared to the other disinfectants (48). In the mentioned study, the Nanosil solution was applied to disinfect meat samples, which caused the bacterial count to reduce on the surface of the samples. In another study, Thomas et al. (1991) investigated the effects of increased pH, sodium chloride, and sodium nitrite on the growth rate of *Salmonella typhimurium*. According to the obtained results, the increased concentration of sodium chloride and decreased pH could inhibit the bacterial growth rate, and bacterial growth was not observed at higher sodium chloride concentrations than 4.8% and pH<4 (48).

According to the results of the present study, the increased concentration of organic acids and duration of microwave radiation caused a significant reduction in the bacterial count logarithm of the meat sections. *P. aeruginosa* growth was not observed after microwave irradiation at the temperature of 65°C for nine seconds. In addition, use of 5% lactic acid significantly reduced the bacterial count logarithm during the refrigeration period, while the increased salt concentration from 4% to 6% and increased microwave duration had no

significant effects on the inhibition of microorganisms during refrigeration.

In the current research, the interactions between lactic acid (2.5% and 5%), salt (4% and 6%), and microwave radiation were investigated, and the obtained results demonstrated that the increased lactic acid concentration from 2.5% to 5% and increased salt concentration from 4% to 6% caused the bacteria to show no growth at the temperatures of 45°C (5 s), 55°C (7 s), and 65°C (9 s). In fact, the combination of microwave radiation, acid, and salt had a greater effect on the bacteria as opposed to the effects of the each factor separately.

In the study by Zeynli et al. (2014), the effects of the combination of various pH levels (4.7, 4.6, and 4.5), acids (lactic, citric, and hydrochloric acid), temperatures (25°C and 35°C), and salt concentrations (3%, 5%, and 6%) on the growth of *S. typhimurium* were investigated on the BHI agar. In addition, the growth rate of the microorganism was assessed considering the observable opacity after 30 days. The obtained results indicated the significant effects of the mentioned parameters on the reduced growth rate of *S. typhimurium* (49).

The most common method for the protection of food products is to lower their pH. Acidifying is considered to be an effective approach at low concentrations to reduce the pathogenic bacteria by reducing the pH (43). In a study re-designed by Le Marc et al. (2008), the effects of temperature, pH and NaCl concentration on the growth of Clostridium perfringens type A were investigated. Treatments with a range of combinations of pH and NaCl concentration are effective (50).

In the present study, the bacterial count of *P. aeruginosa* with 5% lactic acid was significantly lower than 2.5% lactic acid during refrigeration. Several studies have reported that higher temperatures increase the effectiveness of organic acids (51, 52). However, the combined effects of temperature, acid, salt, and other compounds could be influenced by various factors, such as the type of acids and microorganisms (53).

The current research aimed to investigate the combined effects of microwave radiation, lactic acid (2.5% and 5%), and salt (4% and 6%) on *P. aeruginosa* inoculated into veal sections, and the combined effects were observed to be more significant than the individual effects of these parameters. Furthermore, the simultaneous application of these compounds inhibited bacterial growth at lower temperatures, so that the use of 5% lactic acid and 6% salt could simultaneously reduce the bacterial growth at a lower temperature (45°C).

In a similar study, Goncalves et al. (2005) assessed the quantitative effects of chemical treatments on reducing the population of *Listeria monocytogenes* in chicken meat sections. According to the findings, treatment of chicken breasts with 4% lactic acid for 15 minutes at the temperature of 50°C decreased the bacteria by Log 2 CFU/g of the bacteria compared to the control group (54). In the present study, spraying of 5% lactic acid spraying and the combined temperatures of 55°C and 65°C could inhibit the bacterial growth, while the temperature of 55°C only decreased the bacterial growth.

Conclusion

According to the results, simultaneous use of organic acids, salt, and microwave irradiation against *P. aeruginosa* inoculated into the meat sections during refrigeration storage could reduce bacterial growth. Moreover, the interactions between microwave radiation, organic acid, and salt caused further reduction in the bacterial count compared to the control group. A significant difference was also observed in the number of the bacteria between the acid and salt treatments and control group ($P<0.001$).

By controlling the effects of salt concentration and microwave duration, use of 2.5% and 5% lactic acid reduced the bacterial count logarithm during the refrigeration period compared to the acid-free samples ($P<0.001$). In addition, use of 5% lactic acid significantly decreased the bacterial count logarithm during the refrigeration period compared to the samples treated with 2.5% lactic acid ($P<0.001$).

By controlling the impact of the acid concentration and microwave duration, use of 4% and 6% salt significantly decreased the bacterial count logarithm during the refrigeration period compared to the non-salt samples ($P<0.001$). On the other hand, the samples containing 4% and 6% salt had no significant difference in terms of the bacterial count logarithm during the refrigeration period ($P=0.89$).

Finally, microwave duration for nine seconds (65°C) significantly decreased the bacterial count logarithm compared to seven seconds (55°C), five seconds (45°C), and three seconds (30°C) ($P<0.001$). In addition, use of 5% lactic acid and 6% salt caused the microwave duration to reduce from nine seconds (65°C) to five seconds (45°C) in order to complete the elimination of the bacteria.

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Conflict of Interest

None.

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