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Synergistic Antimicrobial Effects of Nisin and ε-Poly-L-Lysine on Raw Beef During Cold Storage Against Major Foodborne Pathogens

Nisin ve ε-Poli-L-Lizin'in Soğuk Depolama Sırasında Çiğ Sığır Etinde Başlıca Gıda Kaynaklı Patojenlere Karşı Sinerjik Antimikrobiyal Etkileri

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ABSTRACT

With increasing consumer demand for natural food preservatives, the use of antimicrobial substances such as nisin and epsilon-poly-L-lysine (ε-PL) in meat products has gained attention. This study aimed to evaluate the synergistic effects of nisin and ε-PL on the viability of key foodborne pathogens—*Escherichia coli* O157:H7, *Salmonella* Typhimurium (Gram-negative), and *Listeria monocytogenes* (Gram-positive)—in raw red meat. In addition, the study evaluated the impact of treatments on physicochemical characteristics and color stability. Two combinations of nisin and ε-PL were tested: Mix 1 (400 IU/g nisin + 20 µg/g ε-PL) and Mix 2 (800 IU/g nisin + 40 µg/g ε-PL), applied to raw beef samples inoculated with the pathogens. Samples were stored at 4 °C for 16 days, and microbiological (pathogen counts, total mesophilic aerobic bacteria, mold, and yeast), physicochemical (pH, water-holding capacity), and color (*L**, *a**, *b**) analyses were performed at intervals (days 0, 4, 8, 12, and 16). Mix 2 showed the most potent antimicrobial activity, decreasing *L. monocytogenes* counts to 2.15 log CFU/g by day 8. Significant reductions were also recorded for *E. coli* O157:H7 and *Salmonella* Typhimurium. Additionally, the mixtures suppressed pH increases, maintained color stability, and improved water retention. In conclusion, the free-form combinations of nisin and ε-PL effectively inhibited microbial growth, preserved meat quality, and extended shelf life, highlighting their potential as natural preservatives in the meat industry. This study provides novel evidence on the synergistic use of free-form nisin and ε-PL in raw beef, offering a valuable contribution to the development of natural preservation methods in meat products.

Keywords: Epsilon-poly-L-lysine (ε-PL), food biopreservation, meat, natural antimicrobials, nisin

ÖZ

Doğal gıda koruyucularına yönelik artan tüketici talebiyle birlikte, et ürünlerinde nisin ve epsilon-poly-L-lysine (ε-PL) gibi antimikrobiyal maddelerin kullanımı dikkat çekmiştir. Bu çalışma, nisin ve ε-PL'nin çiğ kırmızı ette gıda kaynaklı önemli patojenlerin *Escherichia coli* O157:H7, *Salmonella* Typhimurium (Gram-negatif) ve *Listeria monocytogenes* (Gram-pozitif) canlılığı üzerindeki sinerjik etkilerini değerlendirmeyi amaçlamıştır. Çalışmada ayrıca uygulamaların fizikokimyasal özellikler ve renk stabilitesi üzerindeki etkisi de değerlendirilmiştir. Nisin ve ε-PL'nin iki kombinasyonu test edilmiştir: Karışım 1 (400 IU/g nisin + 20 µg/g ε-PL) ve Karışım 2 (800 IU/g nisin + 40 µg/g ε-PL), patojenlerle inoküle edilmiş çiğ sığır eti örneklerine uygulanmıştır. Örnekler 16 gün boyunca 4 °C'de saklanmış ve aralıklarla (0, 4, 8, 12 ve 16. günler) mikrobiyolojik (patojen sayıları, toplam mezofilik aerobik bakteri, küf ve maya), fizikokimyasal (pH, su tutma kapasitesi) ve renk (*L**, *a**, *b**) analizleri yapılmıştır. Karışım 2, *L. monocytogenes* sayısını 8. günde 2,15 log CFU/g'a düşürerek en güçlü antimikrobiyal aktiviteyi göstermiştir. *E. coli* O157:H7 ve *S.* Typhimurium için de önemli azalmalar kaydedilmiştir. Ayrıca, karışımlar pH artışlarını bastırmış, renk stabilitesini korumuş ve su tutma özelliğini geliştirmiştir. Sonuç olarak, nisin ve ε-PL'nin serbest form kombinasyonları mikrobiyal büyümeyi etkili bir şekilde engellemiş, et kalitesini korumuş ve raf ömrünü uzatmıştır.

Anahtar Kelimeler: Doğal antimikrobiyaller, epsilon-poli-L-lizin (ε-PL), et, gıda biyokoruma, nisin

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INTRODUCTION

Meat products are among the most widely consumed foods due to their rich nutritional profile.[1] The constant and increasing demand for meat necessitates an extensive global production and supply chain. Factors such as rapid economic growth, trade liberalization, evolving consumer lifestyles, and increasing food demand have significantly influenced the meat industry, presenting both opportunities and challenges.[1,2] However, practices adopted to meet this growing demand may compromise food safety and increase public health risks.

Due to its high-water activity, protein content, and nutrient richness, meat provides an ideal environment for microbial growth, making it susceptible to physical, chemical, and especially microbiological spoilage.[3] Microbial contamination plays a critical role in determining the safety and shelf life of meat products. During slaughter and processing, contamination can arise from various sources including poor hygiene practices, contaminated tools, animal hides, gastrointestinal contents, and transport conditions.[4] Consequently, raw meat can become easily contaminated with pathogenic bacteria, particularly during the slaughtering process, increasing the risk of serious foodborne illnesses.[1] *Salmonella Typhimurium*, *Listeria monocytogenes*, and Shiga-toxin-producing *Escherichia coli* O157:H7 are among the most implicated pathogens associated with raw red meat.[5,6] Although the food industry has invested heavily in measures to control these microorganisms, they continue to pose significant public health threats. Therefore, innovative and more effective strategies are needed to address these ongoing microbiological challenges.

Nisin, a bacteriocin produced by *Lactococcus lactis*, has received Generally Recognized as Safe (GRAS) status from the U.S. Food and Drug Administration (FDA).[7] It is widely used as a natural preservative, particularly in meat and dairy products, due to its potent antimicrobial activity, especially against Gram-positive bacteria such as *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum*.[7,8]

Epsilon-poly-L-lysine (ε-PL) is a naturally occurring cationic peptide composed of L-lysine residues and is produced by fermentation using *Streptomyces albulus*. It is water-soluble, biodegradable, non-toxic, and exhibits high thermal stability. ε-PL has demonstrated broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, including *Bacillus subtilis*, *E. coli*, lactic acid bacteria, and *Staphylococcus aureus*.[9] Its mechanism of action involves binding to negatively charged bacterial cell membranes due to its cationic nature, leading to membrane disruption and cell death.[10] This unique mechanism allows ε-PL to be effective against a wide range of bacterial pathogens.

When used in combination with nisin, ε-PL has shown enhanced antimicrobial effects. Literature reports suggest that nisin + ε-PL combinations are particularly effective against Gram-positive pathogens such as *B. cereus*, *L. monocytogenes*, *S. aureus*, and *Enterococcus faecalis*, as well as some Gram-negative species including *B. subtilis* and *Lactobacillus* spp.[11] While earlier studies[12] did not report synergistic activity of this combination against *E. coli*, more recent work by[13] demonstrated promising antimicrobial effects on this pathogen. These findings underscore the need for further research to better understand the efficacy of nisin and ε-PL combinations, particularly against Gram-negative bacteria.

In this study, we aimed to evaluate the synergistic activity of nisin and ε-PL against key foodborne pathogens, specifically Gram-negative (*E. coli* O157:H7, *S. Typhimurium*) and Gram-positive (*L. monocytogenes*) bacteria, in raw red meat. Additionally, we assessed the impact of these combinations on the physicochemical and color properties of the meat.

MATERIALS AND METHODS

Ethics Committee

In accordance with Article 8(k) of the 'Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees', this study is not subject to HADYEK permission.

Preparation of mixtures of nisin and epsilon-poly-L-lysine (ε-PL)

Cationic nisin and ε-poly-L-lysine (ε-PL) were obtained commercially from Handary (Belgium). Stock solutions of nisin (0.5 mM; 1.68 mg/mL) and ε-PL (5 mM; 0.72 mg/mL) were prepared using HEPES buffer (5 mM, Sigma, UK) with low ionic strength. These solutions were subsequently filtered through 0.45 µm syringe filters to ensure sterility. To simulate the conditions of raw meat, the pH of the solutions was adjusted to 5.5, matching the typical pH level of fresh beef. Two different combinations of nisin and ε-PL were formulated: Mix 1: 400 IU/g nisin + 20 µg/g ε-PL, and Mix 2: 800 IU/g nisin + 40 µg/g ε-PL. Each mixture was stirred continuously for 4 hours using a magnetic stirrer to ensure complete homogenisation. The prepared antimicrobial solutions were subsequently applied directly to the surface of the meat samples.

Preparation of Pathogenic Bacterial İnoculum

The bacterial strains used to inoculate the meat samples included *Escherichia coli* O157:H7 (ATCC 43984), *Salmonella* Typhimurium (ATCC 14028), and *Listeria monocytogenes* (RSKK 474, 476), all obtained from the Refik Saydam National Public Health Agency (Turkey). Each strain was cultured in Tryptic Soy Broth (TSB) at 37°C for 18–24 hours. Following incubation, bacterial cells were collected by centrifuging at 4,000 rpm for 10 minutes. The obtained cell pellets were then rinsed with 0.1% peptone water to eliminate any remaining culture medium. Pellets belonging to the same species were pooled and in sterile physiological saline to prepare a uniform inoculum. Serial dilutions were prepared to obtain an inoculation level of approximately 10^5 CFU/g for each target pathogen in the meat samples.

Preparation of the Groups

Musculus longissimus dorsi from cattle slaughtered under hygienic conditions one day prior and having undergone rigor mortis was used as the meat source in this study. The meat was obtained from local butchers in Şanlıurfa Province and transported to the Food Hygiene and Technology Laboratory at Harran University, Faculty of Veterinary Medicine, under cold chain conditions. A total of 30 meat samples (15 per replicate) were used. The meat was aseptically cut into small pieces (25 ± 5 g) using a sterile scalpel. The meat samples were then experimentally inoculated with the diluted bacterial suspension. Specifically, 500 µL of the pathogen cocktail was uniformly spread across each meat sample using a sterile spreader, and samples were held for at least 10 minutes to facilitate bacterial attachment. The samples were randomly assigned into three groups: Control group (no treatment), Mix 1 (400 IU/g nisin + 20 µg/g ε-PL), Mix 2 (800 IU/g nisin + 40 µg/g ε-PL).

Each sample was placed into a 50 mL falcon tube. Then, 500 µL of the respective antimicrobial solution (prepared in HEPES buffer) was added. The tubes were shaken gently for 2 minutes to ensure uniform distribution of the treatment. After treatment, all samples were stored at 4 ± 1°C for 16 days. Microbiological, chemical, and instrumental color analyses were performed at 4-day intervals (days 0, 4, 8, 10, 12, and 16). The experiment was conducted in two independent replicates.

Microbiological Analyses

On each analysis day, meat samples were aseptically transferred from falcon tubes into sterile stomacher bags. A volume of 225 mL of 0.1% peptone water (Merck, Darmstadt, Germany) was added to each bag, and the mixture was homogenized using a stomacher for 3 minutes to obtain a 10^{-1} dilution. Serial dilutions were then prepared up to 10^{-7} using the same diluent. For microbial enumeration, the surface spread method was used for *E. coli* O157:H7, *L. monocytogenes*, and *S. Typhimurium*, while the pour plate method was employed for the total mesophilic aerobic bacteria (TMAB), molds, and yeasts. All inoculations were performed in duplicate. After incubation, microbial colonies were enumerated based on their characteristic morphology on selective media, in accordance with guidelines.[14]

Listeria monocytogenes; Enumerated on Oxford Agar (Merck, Darmstadt, Germany). After incubation at 35°C for 24–48 hours, colonies with a blackish-green to brown color, featuring black zones and sunken centers, were counted. *Salmonella Typhimurium*; Counted on Xylose Lysine Deoxycholate (XLD) Agar (Merck, Darmstadt, Germany). Colonies appearing black after incubation at 35°C for 24–48 hours were considered presumptive *S. Typhimurium*. *Escherichia coli* O157:H7; Counted using Cefixime Tellurite Sorbitol MacConkey (CT-SMAC) Agar (Merck, Darmstadt, Germany). White colonies were counted after incubation at 35°C for 24–48 hours. Total mesophilic aerobic bacteria (TMAB); Enumerated using Plate Count Agar (Merck, Darmstadt, Germany). The inoculated plates were incubated at 35°C for 24–48 hours prior to colony enumeration. Molds and yeasts; Enumerated on Dichloran Rose Bengal Chloramphenicol (DRBC) Agar (LAB 217; Lab M, Lancashire, UK). Plates were incubated at 25 ± 1°C for 5 days in accordance with ISO 21527-1.[15]

Physicochemical Analyses

pH Determination

pH measurements were conducted at 25°C using a digital pH meter (model HI 11310, Hanna Instruments, USA). Prior to each measurement, the instrument was calibrated using standard buffer solutions at pH 4.01 and 7.00. For analysis, 10 g of the meat sample was combined with 90 mL of distilled water and homogenized for 1 minute. The resulting mixture was then used for pH measurement.[16]

Water Holding Capacity

Approximately 2 g of each meat sample was placed between filter papers and placed between two glass plates (10 × 10 cm). A 10 kg weight was applied to the setup for 5 minutes. The water holding capacity was then calculated using the following equation:

$$WHC(\%)=100-[(\text{First weight}-\text{Last weight})/\text{First weight}]x100$$

Color Analysis

The color characteristics of the meat samples were determined using a digital colorimeter (model CS-10, CHNSpec, Hangzhou, China). Lightness (L^*), redness (a^*),

and yellowness (*b**) values were measured at a minimum of four different points on the outer surfaces of the samples to ensure accuracy. Prior to analysis, the colorimeter was calibrated using standard black and white reference plates.[17]

Statistical Analysis

The data obtained from the study were analysed using SPSS 24.0 (IBM Corp., Armonk, NY, USA). Microbial counts, pH, water holding capacity and colour parameters (L*, a*, b*) were subjected to statistical analysis. One-way analysis of variance (ANOVA) was employed to evaluate differences among groups and sampling days. To identify statistically significant differences, a Tukey's post hoc test was performed. All measurements were performed in duplicate in independent trials, and the data are presented as mean values accompanied by the standard error of the mean (± SE). A significance level of $P < .05$ was considered statistically significant.

RESULTS

In this study, the effects of mixtures containing different ratios of nisin and ε-PL (Mix 1 and Mix 2) on the microbiological, physicochemical, and color parameters of raw beef stored at 4 °C were evaluated. The results demonstrated that these mixtures exhibited significant antimicrobial activity.

Microbiological Results

The counts of *Listeria monocytogenes* did not show a significant change in the control group during storage ($P > .05$). However, both Mix 1 and, in particular, Mix 2 treatments exhibited a significant reduction in bacterial load. A 1-log difference between the control and treatment groups was observed on the 4th day, and an approximately 3-log difference was noted on the 8th day, with this decrease being statistically significant ($P < .05$; Table 1). Although a slight increase in the number of *L. monocytogenes* was observed in the treatment groups toward the end of storage, the difference remained statistically significant ($P < .05$), resulting in an approximately 2-log reduction relative to the control. The concentrations of nisin and ε-PL in the treatment groups did not significantly influence the inhibition of *L. monocytogenes*.

Table 1. *Listeria monocytogenes* counts (log 10 cfu/g±SE) in raw beef during storage at 4°C.a

	Storage time (days)				
Concentrations	0.	4.	8.	12.	16.
Control	5.32±0.04	$5.62±0.10^{a}$	$5.60±0.15^{a}$	$5.62±0.18^{a}$	$5.57±0.15^{a}$
Mix 1	$5.42±0.05^{A}$	$4.49±0.20A^{Bb}$	$2.65±0.24^{Cb}$	$3.66±0.22^{BCb}$	$3.72±0.19^{BCb}$
Mix 2	$5.24±0.13^{A}$	$4.30±0.23A^{Bb}$	$2.15±0.15^{Cb}$	$3.45±0.27^{Bb}$	$3.57±0.23^{Bb}$

a-b: Mean values shown with different letters in the same column are significantly different ($P < .05$). A-C: Mean values indicated by different letters in the same row are significantly different ($P < .05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

Similarly, significant decreases in *E. coli* O157:H7 counts were observed in the Mix 1 and Mix 2 groups compared to the control group, particularly on days 8, 12, and 16 ($P < 0.05$). At the end of the 16th day, the *E. coli* O157:H7 level in the Mix 2 group was 4.82 ± 0.17 log cfu/g, which represented a difference of approximately 1 log compared to the control group (Table 2). Regarding the concentrations of nisin and ε-PL in the treatment groups, it was noted that the Mix 2 group was more effective in reducing *E. coli* O157:H7 counts, particularly on days 12 and 16.

Table 2. Escherichia coli O157:H7 counts (log 10 cfu/g±SE) in raw beef during storage at 4°C.

	Storage time (days)				
Concentrations	0.	4.	8.	12.	16.
Control	5.51±0.14	5.52±0.17	$5.53±0.27^{a}$	$5.74±0.18^{a}$	$5.84±0.15^{a}$
Mix 1	$5.67±0.10^{A}$	$5.72±0.22^{A}$	$4.83±0.12^{Bb}$	$5.50±0.10^{Aab}$	$5.19±0.12^{ABab}$
Mix 2	$5.65±0.16^{A}$	$5.47±0.26^{A}$	$4.90±0.16^{Bb}$	$5.10±0.26^{ABb}$	$4.82±0.17^{Bb}$

a-b: Mean values shown with different letters in the same column are significantly different ($P < .05$). A-B: Mean values indicated by different letters in the same row are significantly different ($P < .05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

No significant decrease in *Salmonella* count was observed in the control group; however, statistically significant reductions were recorded in the Mix 1 and Mix 2 groups throughout the entire storage period ($P < .05$). Significant decreases were particularly evident in the Mix 1 and Mix 2 groups compared to the control group, especially on days 4, 8, 12, and 16 ($P < .05$). On day 4, the Mix 2 group exhibited the lowest value at 4.55 ± 0.22 log cfu/g (Table 3). Regarding the concentrations of nisin and ε-PL in the treatment groups, it was observed that the Mix 2 group was more effective in reducing *S.* Typhimurium counts, but only on day 16.

While TMAB counts increased over time in the control group (6.62 ± 0.35 log cfu/g), the increase was more limited in the Mix 1 and Mix 2 groups, with statistically significantly lower values recorded on all days compared to the control group ($P < .05$; Table 4). The concentrations of nisin and ε-PL did not result in significant differences in TMAB counts within the treatment groups ($P > .05$). In contrast to the control group, TMAB counts in the treatment groups remained at day 0 levels even on day 16.

While mold and yeast counts increased significantly in the control group, the increase was slower in the Mix 1 and, especially, the Mix 2 groups. Notably, the Mix 2 group reached the lowest level of 1.23 ± 0.23 log cfu/g on the 8th day (Table 5).

Table 3. *Salmonella* Typhimurium counts (log 10 cfu/g±SE) in raw beef during storage at 4°C.

	Storage time (days)				
Concentrations	0.	4.	8.	12.	16.
Control	5.75±0.04	$5.40±0.18^{a}$	$5.47±0.30^{a}$	$5.57±0.18^{a}$	$5.37±0.19^{a}$
Mix 1	5.29±0.08A	$4.84±0.10^{Bb}$	$4.97±0.20^{Bb}$	$4.92±0.13^{Bb}$	$5.24±0.14^{Aa}$
Mix 2	$5.34±0.06^{A}$	$4.55±0.22^{Bb}$	$4.72±0.15^{Bb}$	$4.88±0.17^{Bb}$	$4.90±0.12^{Bab}$

a-b: Mean values shown with different letters in the same column are significantly different ($P <.05$). A-B: Mean values indicated by different letters in the same row are significantly different ($P <.05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

Table 4. Total mesophilic aerobic bacteria (TMAB) count (log 10 cfu/g±SE) in raw beef during storage at 4°C.

	Storage time (days)				
Concentrations	0.	4.	8.	12.	16.
Control	$5.55±0.11^{B}$	$5.91±0.06^{ABa}$	$6.19±0.15^{Aa}$	$6.48±0.18^{Aa}$	$6.62±0.35^{Aa}$
Mix 1	$5.54±0.10^{A}$	$4.97±0.16^{Bb}$	$5.58±0.14^{Ab}$	$5.80±0.11^{Ab}$	$5.42±0.27^{Ab}$
Mix 2	$5.40±0.15^{A}$	$4.93±0.23^{Bb}$	$5.42±0.15^{Ab}$	$5.92±0.25^{Ab}$	$5.70±0.25^{Ab}$

a-b: Mean values shown with different letters in the same column are significantly different ($P < .05$). A-B: Mean values indicated by different letters in the same row are significantly different ($P < .05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

Table 5. Mold and yeast count (log 10 cfu/g±SE) in raw beef during storage at 4°C.

	Storage time (days)				
Concentrations	0.	4.	8.	12.	16.
Control	$1.27±0.14^{B}$	$2.38±0.21^{Aa}$	$2.60±0.27^{Aa}$	$2.45±0.15^{Aa}$	$2.40±0.25^{Aa}$
Mix 1	$1.71±0.13^{A}$	$1.23±0.23^{Bb}$	$1.76±0.33^{Ab}$	$1.87±0.17^{Ab}$	$1.97±0.23^{Aa}$
Mix 2	$1.66±0.26^{A}$	$1.20±0.20^{Bb}$	$1.23±0.23^{Bc}$	$1.77±0.20^{Ab}$	$2.03±0.28^{Aa}$

a-b: Mean values shown with different letters in the same column are significantly different ($P <.05$). A-B: Mean values indicated by different letters in the same row are significantly different ($P <.05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

Physicochemical Results

In the control group, the pH value increased over time, reaching 6.40 ± 0.19 by the end of the 16th day ($P <.05$). In contrast, pH changes were less pronounced in the Mix 1 and Mix 2 groups, and a significant difference was observed between the control and treatment groups on day 16 ($P <.05$, Figure 1). This suggests that the applied mixtures effectively suppressed microbial activity and prevented pH increases. No significant differences in water holding capacity were observed between the groups throughout the storage period ($P >.05$), nor were there any significant differences between the treatment groups ($P >.05$; Figure 1).

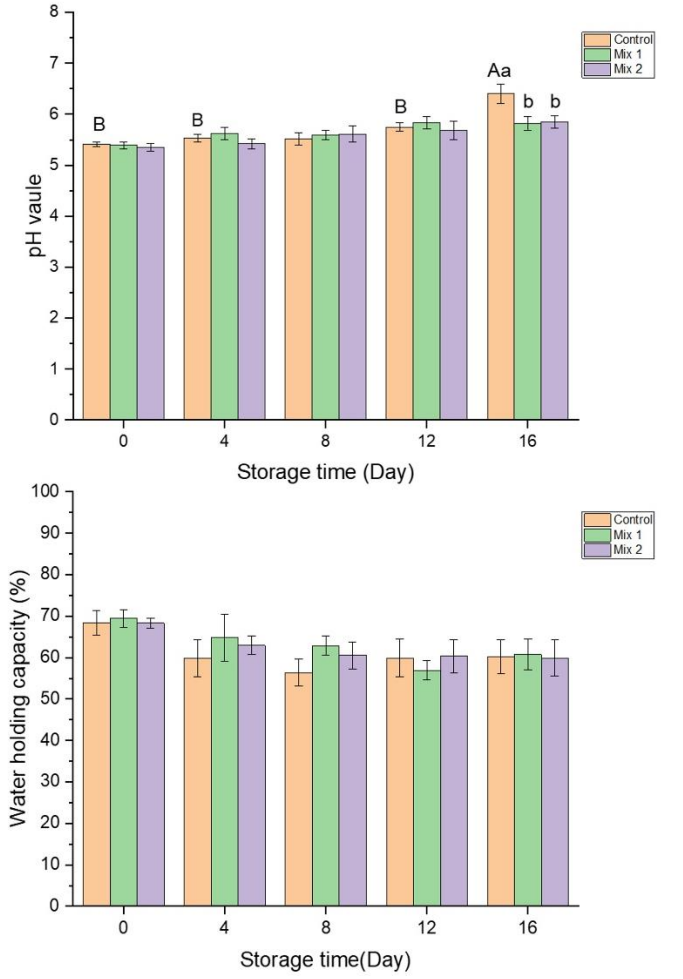

Figure 1. pH value and water holding capacity of raw beef during storage at 4 °C (Mean ±SE). a-b: Mean values indicated by different letters between groups, A-B: Mean values indicated by different letters between sampling days are significantly different ($P < .05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

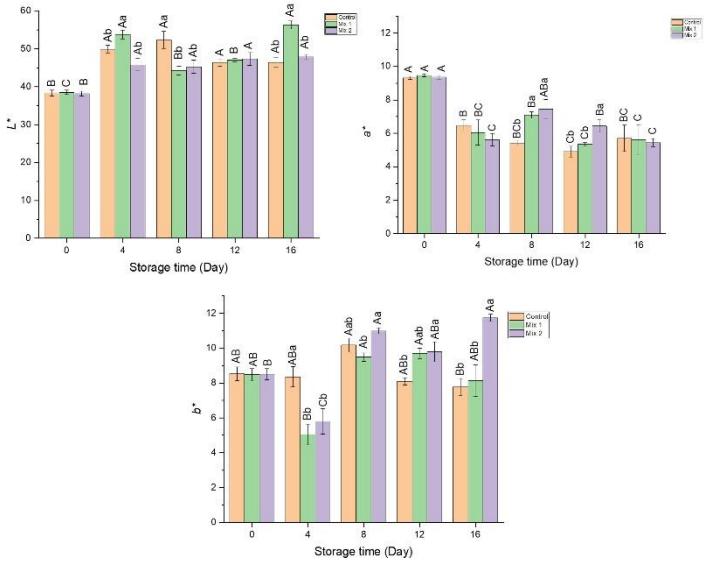

Figure 2. Color values of raw beef during storage at 4°C (Mean ±SE). a-b: Mean values indicated by different letters between groups, A-C: Mean values indicated by different letters between sampling days are significantly different ($P < .05$). Mix 1: 400IU/g nisin + 20µg/g, ε-PL Mix 2: 800IU/g nisin + 40µg/g, ε-PL

Color Parameters

In terms of the *L** value, a significant increase (53.79 ± 1.16) was observed, particularly on day 4 in the Mix 1 group, compared to the control group (P <.05). The *a** value decreased over time, but it remained higher in the Mix 2 group on day 8 (7.44 ± 0.58) compared to the control group (P <.05; Figure 2). The *b** value was significantly higher in the Mix 2 group on days 8 and 16 (e.g., day 16: 11.73 ± 0.20, P <.05; Figure 2). These results suggest that the combination of nisin and ε-PL may positively influence the color stability of meat.

DISCUSSION

In the present study, the application of combined nisin and ε-PL treatments, particularly Mix 2 (800 IU/g nisin + 40 µg/g ε-PL), significantly reduced the counts of *Listeria monocytogenes* in raw beef, reaching 2.15 log cfu/g by day 8. This result aligns with the findings of Zimet et al.[18], who demonstrated that free-form nisin effectively reduced *L. monocytogenes* levels in lean beef. The slight increase in *L. monocytogenes* numbers in the treatment groups towards the end of storage can be attributed to the fact that free-form nisin and ε-PL are effective for a limited period. As noted in previous studies, free nisin is influenced by the food matrix, and significant decreases in its activity and stability are observed when applied in its free form to food.[19]

The data obtained for *E. coli* O157:H7 once again demonstrated the limited effect of nisin against Gram-negative bacteria, while ε-PL partially compensated for this limitation. In the Mix 2 group, the counts decreased to 4.82 log cfu/g by day 16, whereas the decrease in Mix 1 was more limited. This difference is likely due to the lower concentrations of nisin and ε-PL in Mix 1, which resulted in a more limited duration of action and reduced stability in the meat matrix.[19] Furthermore, although ε-PL is known to be an effective antimicrobial agent against Gram-negative bacteria, no significant reduction in *E. coli* O157:H7 was observed, which may be due to the insufficient concentration of ε-PL applied.

The effects on *S.* Typhimurium were evaluated similarly. Although a significant decrease (4.55 log cfu/g) was achieved in the early period (day 4) in the Mix 2 application, the continuity of this effect was limited in the following days. This reduction in effectiveness may be due to interactions between free-form antimicrobials and food matrix components, which reduce their stability and bioavailability. Similar to the results observed for *E. coli* O157:H7, both free nisin and free ε-PL demonstrated antimicrobial activity against *S. Typhimurium*, another

Gram-negative pathogen in meat samples, with a reduction in Salmonella count of approximately 1 log in the first 8 days of preservation. However, this effect was not observed in the remaining days, likely due to the decrease in the stability of these antimicrobials over time.

In the control group, pathogenic bacteria counts appeared relatively stable throughout the storage period. This observation can be explained by several intrinsic factors associated with raw beef. Cold storage at 4±1°C is known to suppress bacterial growth and delay the proliferation of pathogens by slowing down metabolic activity and prolonging the microbial lag phase.[20] In addition, the presence of natural microflora in raw meat, particularly lactic acid bacteria (LAB), may have exerted a slight antagonistic effect on inoculated pathogens. These naturally occurring microorganisms may compete for essential nutrients and surface attachment sites or produce inhibitory compounds such as organic acids and bacteriocins, thereby limiting the growth of exogenous pathogens.[21] Although background flora was not quantitatively analysed in this study, it is possible that microbial interference contributed to the observed stabilization of pathogen populations in untreated control samples, especially in the early stages of storage.

The literature provides various reports on the effectiveness of nisin and ε-PL against important foodborne pathogens. These antimicrobials are shown to be effective at different levels.[22-27] The variations in effectiveness between studies can be attributed to factors such as bacterial strain, application time, method, antimicrobial concentration, and food type. Most notably, the antibacterial activity of these antimicrobials may be influenced by the composition of the food matrix. In fact, a previous study[22] reported that ε-PL exhibited a more pronounced bacteriostatic effect in rice and vegetable extracts than in milk, beef, or sausage extracts, which are rich in protein content. Furthermore, it has been reported that the antibacterial effect of ε-PL can be altered by its interaction with food components, forming a compound with a different charge, which reduces its ability to interact with anionic microbial surfaces and diminishes its antibacterial activity against *E. coli*.[28] Therefore, while nisin and ε-PL in free form are effective against foodborne pathogens, their efficacy is limited. To enhance their effectiveness in food applications, they should be supported by encapsulation technologies that enable controlled release systems.

The acceptable upper limit for total viable counts in meat and meat products is generally considered to be around 7.0 log10.[20] While TMAB counts in the control group approached this limit on day 12 (6.62 log cfu/g), they remained significantly lower in the Mix 1 and Mix 2 groups. These findings align with similar studies; in particular, it has been reported that ε-PL exhibited a strong antimicrobial effect in meat and meat products, significantly reducing TMAB counts.[26,29] In contrast, a reduction of around 0.5 log CFU/g in TMAB counts was observed in sausages packaged with nisin activity.[26] In Mix 1 and Mix 2 groups, TMAB counts decreased on day 4 and then increased again (Table 4). These fluctuations were also observed in mesophilic pathogenic bacteria (*S.* Typhimurium and *E. coli* O157:H) (Table 2, Table 3). However, such a biphasic growth pattern is not unusual and may be explained by microbial stress adaptation dynamics. Immediately after cold storage and exposure to antimicrobials, bacterial cells can enter a state of cold shock or non-lethal injury, which temporarily reduces their culturability. As storage progresses and the bacteria adapt to the cold environment and antimicrobials, the injured cells can repair their membranes and enzyme systems, re-enter active growth and thus rise again at later stages.

Meat and meat product surfaces are particularly vulnerable to contamination by molds and yeasts, leading to deterioration in both quality and sensory characteristics. In the present study, it was observed that the antimicrobial treatments reduced the number of yeasts and molds in the treatment groups until the 8th day of preservation. This result supports the antifungal potential of the antimicrobial mixtures. The findings are consistent with studies showing that polyethylene films combined with nisin (400-800 IU/g) exhibit significant antimicrobial activity against yeasts and molds in cutlets during storage[30], and that nisin and ε-PL have notable antimold activity in packaged sausages.[26]

When the pH values were analysed, significant increases were observed in the control group by the 16th day (6.40), while the pH values remained stable around 5.8 in the Mix 1 and Mix 2 groups. The higher pH in the control sample can be attributed to bacterial growth, particularly the production of lactic acid by lactic acid bacteria, as well as the inhibition of protein degradation and the formation of nitrogenous compounds by the antimicrobial agents, which suppress microbial activity.[31,32] In fact, TMAB numbers increased significantly in the control group compared to the other groups (Table 5). The increase in bacterial population leads to a rise in bacterial enzyme activity in the meat tissue, where these enzymes break down meat proteins and produce nitrogenous compounds, ultimately causing an increase in pH.[32] Although there was no statistically significant difference between the groups in terms of water holding capacity (WHC), it is noteworthy that higher values were recorded in the Mix 1 group on the 4th and 8th days. This suggests that the applied antimicrobial mixture may

indirectly help preserve the water holding capacity of meat by reducing protein denaturation.[33] The more stable pH values also support this outcome, as water loss tends to increase when proteins approach their isoelectric point.[34]

When the color parameters were examined, the values for *L**, *a** and *b** varied during storage. In terms of *L** values, it was observed that the Mix 1 application contributed to the formation of lighter colored meat, especially in the initial period. The *a** (redness) value was highest in the Mix 2 group on the 8th day (7.44); this could be linked to the suppression of lipid oxidation and the delayed formation of metmyoglobin. A decrease in *a** values is often considered an indicator of myoglobin oxidation and metmyoglobin formation, which causes the meat to turn brown.[35] On the 8th and 12th days, the *a** value in the treatment groups was higher than in the control group (Figure 2), demonstrating the antimicrobials' ability to delay the formation of metmyoglobin and thus preserve the color quality of the meat for a longer period.[31] The *b** value also increased significantly in the Mix 2 group, with the treatment groups showing higher *b** values than the control, particularly towards the end of the preservation period (Figure 2). This may be related to the antimicrobial effects of the treatment. A decrease in the *b** value is typically associated with reduced oxymioglobin content and increased metmyoglobin formation, with the decline in oxymioglobin attributed to oxygen consumption by microorganisms.[35] Additionally, this increase in *b** value could be due to the color changing properties of the antimicrobials themselves. Indeed,[26] emphasized that the high *b** value in sausages was due to the yellow color of the antimicrobial agents used.

In conclusion, this study evaluated the effects of free-form combinations of the natural antimicrobial agents nisin and ε-PL on major foodborne pathogens, including *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* Typhimurium. The findings demonstrated that the combination of nisin and ε-PL in free form exhibited significant antimicrobial activity when applied in specific ratios. Notably, the treatments effectively suppressed *L. monocytogenes*, with Mix 2 (higher concentration) showing considerable activity against *E. coli* O157:H7 and *S.* Typhimurium by the end of the storage period. In addition, the antimicrobial mixtures helped to limit pH increases, preserve water holding capacity, and maintain stable color characteristics in the meat. These results suggest that nisin and ε-PL, when combined in appropriate ratios, can delay microbial spoilage and help maintain the quality of raw meat products. However, to prolong their effectiveness, these compounds should be coupled with encapsulation technologies. Future research on their application across various meat types, alongside packaging solutions, consumer acceptance, and sensory evaluations, will provide further insights into their potential for enhancing food safety and quality.

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