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Antimicrobial Activity of Nisin and Lysozyme on Foodborne Pathogens Listeria Monocytogenes, Staphylococcus Aureus, Salmonella Typhimurium, and Escherichia Coli at Different pH

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ABSTRACT

Background: To prevent or inhibit the growth of pathogenic microorganisms and food spoilage factors, many studies have been done by using natural preservatives. The aim of study was to investigate the effect of different concentrations of lysozyme and Nisin on the growth rate and also to determine the minimum inhibitory concentration (MIC) and minimum bactericidal cocentratiin (MBC) of these combinations on the bacteria of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimorium* and *Listeria monocytogenes.* **Methods:** In this study, various concentrations of lysozyme and Nisin were set in form of alone concentration and in combination concentrations (0, 19.53, 39.06, 78.13, 156.25, 312.5, 625, 1250, 2500, 5000) in vitro conditions and 6 pH 5.5, 6, 6.5, 7, 7.5, and 8. Microdilution method at 24°C was done and the combined effect on bacteria growth was read by using ELISA reader. **Results:** The results showed that lysozyme was less effective on *Escherichia coli* and Nisin was less effective on *Listeria monocytogenes*. Moreover, combining lysozyme and Nisin at low pH decreased the MIC. **Conclusions:** The results of the study showed that the effect of combining lysozyme and Nisin on *Staphylococcus aureus* is above all other bacteria and at low pH reduces the MIC.

Keywords: *Antibacterial effect; MBC; MIC; Natural preservative*

Introduction

Due to adverse effects of industrial chemicals and their carcinogenicity and toxicity for humans, the debates have increased on using natural preservatives.

To meet this end, food industries have interested in using antimicrobial preservatives that are perceived as more "natural". However, many natural antimicrobials have a limited spectrum of activity and are effective only at high concentrations. A possible solution may use combinations of these antimicrobials (Sofos *et al.*, 1998).

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There has been a great interest within the food industry during the last decade in using enzymes naturally occurring in foods, such as lysozyme (Ntzimani *et al.*, 2010). Lysozyme occurs in a number of animal secretions and is considered as an important component of the innate immune system (Zhang *et al.*, 2006). Lysozyme has high potential in food preservation due to its stability over a wide range of pH and temperatures (Proctor and Cunningham, 1988). Lysozyme is a lytic enzyme found in foods, such as milk and eggs. It is a muraminidase that hydrolyses β1–4 linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan layer of the bacterial cell wall. Given that Gram-negative bacterial cell walls are protected by an outer membrane, lysozyme does not show antibacterial activity against these species (Nattress and Baker, 2003). It is known to inhibit some Gram-positive bacteria however, it alone is ineffective against Gram-negative bacteria. Lysozyme has only limited applications in the food industry and is added to certain hard and semi-hard cheeses in Europe to prevent gas formation by butyric fermentation clostridia, especially *Clostridium tyrobutyricum*. Other potential applications are including its use in heat-sterilized products, its inclusion in immobilized enzyme columns to prevent contamination, and its use as a preservative (Sofos *et al.*, 1998).

Nisin is generally recognized as safe for being used as a food additive in many countries and is approved as food preservative in over 50 countries (Jay, 2000). Nisin is a bacteriocin produced by *Lactococcus lactis* subsp. Lactis, is a low molecular weight antimicrobial protein (Holzapfel *et al.*, 1995). Nisin acts mainly as a membrane depolarizing agent in a voltage dependent fashion (Delves-Broughton, 1990). It is a broad inhibitory spectrum against many Gram-positive bacterial and their spores; however, it shows little or no activity against Gram negative bacteria, yeasts or moulds (Delves-Broughton *et al.*, 1996, Hurst, 1981). Nisin is stable under refrigerated storage of foods, demonstrates heat stability, and is degraded in the gut system. The antimicrobial activity of Nisin against bacteria in foods could be improved by the combined addition with other antimicrobial agents, such as chelators (Murdock *et al.*, 2007) or plant essential oil (Solomakos *et al.*, 2008).

Materials and Methods

Bacterial strain: To use standard strains of foodborne bacteria and compare the effect of lysozyme and Nisin on Gram-positive and Gram negative bacteria, *Listeria monocytogenes* American Type Culture Collection 1143 (ATCC) and *Staphylococcus aureus* Persian Type Culture Collection 1133 (PTCC) (obtained from the Pasteur Institute of Iran) were prepared as Gram-positive bacteria. *Salmonella typhimurium* Razi Type Culture Collection 1735 (RTCC) and *Escherichia coli* 2310 (obtained from the Razi Vaccine and Serum Research Institute, Iran) as Gram-negative bacteria. All cultures were maintained on trypticase soy agar (TSA; BBL, Becton Dickinson, FranklinLakes, NJ) at 37°C. They were kept under refrigeration conditions and re-cultivated at intervals of three to four weeks to maintain activity. To obtain single colony from suspension of bacteria, a linear culture on nutrient agar was performed. In the next step, three single colonies from each bacterium cultured in test tubes containing 5 ml of Trypticase Soy Broth (TSB; Difco, Sparks, MD). Working cultures were obtained by inoculating a loop of pure culture into TSB and incubating at the optimum temperature for each strain for 24 h.

Preparation of solutions:

- Preparation of lysozyme solution

Lysozyme from chicken egg albumen (Sigma-Aldrich, USA), having an activity of about 70,000 U/mg, and lysozyme powder dissolved in sterilized distilled water and sterilized by microbiological filter of 0.45µ. The concentrations of lysozyme were zero, 19.53, 39.69, 13.78, 25/156, 312, 625, 1250, 2500, 5000 µg/mL. The dilution method was adapted from that of Maclean *et al*. (Maclean *et al.*, 1997a, 1997b).

- Preparation of Nisin solution

Nisin powder (Sigma-Aldrich, USA) dissolved in sterilized distilled water and sterilized by microbiological filter of 0.45µ. The concentrations of

Nisin were zero, 19.53, 39.69, 13.78, 25/156, 312, 625, 1250, 2500, 5000 µg/mL. The dilution method was adapted from of the study of Maclean *et al.* (Maclean *et al.*, 1997a, 1997b).

Determination of minimum inhibitory concentration (MIC): A micro-broth dilution assay was used to evaluate the antimicrobial efficacy of lysozyme and Nisin against *L. monocytogenes*, *S. typhimurium*, *E. coli*, and *S. aureus*. Sterile 96-well polystyrene microtitre plates with well capacities of 300 µl were used (Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, USA) and 150 µL of TSB was added to each well of the plate except for the first column. The pH of the medium were adjusted to 5.5, 6, 6.5, 7, 7.5 and 8 (Alexander and and Richard, 2003). Lysozyme and Nisin stock solutions at 10000 µ/mL were filter-sterilized by passage through a 0.45 µm membrane filter (Corning Incorporated, Corning, NY, USA). Then, 150 microlitres of the stock solutions were added to each well of the first column using a multi-channel pipettor (Eppendorf, Hamburg, Germany). After that, 150µl of the stock solution was removed from the first column and mixed thoroughly with the broth in the corresponding wells of the second column six times. Subsequently, a 150 µL aliquot was removed from each well in this column and mixed with the corresponding well of the next column. This doubling dilution was performed in rows across the plate except for the last column that was kept for controls. As a result of such dilution, the gradient of lysozyme and Nisin concentrations obtained from 0 to 10 mg/mL across the plate. Ten microlitres of bacterial culture after 24h incubation were inoculated in each well of the plate to yield a final concentration of 10^5 CFU (Colony Forming Unit)/mL. Bacterial growth was measured by a change in absorbance at 630 nm using ELISA reader (AWARENESS Technologic, USA). The MIC was determined as the lowest lysozyme and Nisin concentration that resulted in inhibition of bacterial growth (lack of increase in absorbance reading). Fifty microlitres of the contents in those wells was also spotted on TSA for confirming the inhibition (absence of growth). The MIC assay was carried out in duplicate for each bacteria and the assay was also repeated three times on different occasions (Alexander and and Richard, 2003).

In the second step, the activity of lysozyme in concentrations and the above pH at 24°C were evaluated by ELISA reader. To determine the MBC, bacterial colonies were counted on MH medium (Zhang *et al.*, 2006).

Data analysis: Statistical analysis was carried out using Sigma Stat (SPSS science, Version 2.0, 1997, Chicago, IL, USA) and one-way variance (Hansen, 1994).

Results

For Nisin, the function was 8 (**Figure 1**); however, the effect of lysozyme on *E. coli* did not show a significant reduction in bacterial growth (**Figure** 2) ($P < 0.05$). The combined effects of Nisin and lysozyme at pH 8 and 7.5 were the best performances ($P < 0.05$) (**Figure 3**). The effect of Nisin on *L. monocytogenes* at high pH, especially 7.5, was the best performance ($P < 0.05$) (**Figure 4**). As shown in **Figure 5**, all pH showed less bactericidal absorption than control of *L. monocytogenes*; however, they did not inhibit the growth of bacteria. The pH of 5.5 was better than other pH ($P < 0.05$).According to **Figure 6**, the absorption of bacteria was observed at initial dilutions of all pH. The pH of 5.5 was better than other pH for the combined effect of Nisin and lysozyme ($P < 0.05$).Regarding *S. typhimurium*, Nisin was better at high pH; however, lysozyme had a negligible effect ($P < 0.05$) (**Figure 7 and 8**). According to **Figure 9**, the combination of Nisin and lysozyme in all dilutions of the pH 7 and 8, except dilution of 19.53 µg/mL, inhibited *S. typhimurium* growth. The pH were better for the combined action of Nisin and lysozyme ($P < 0.05$). The effect of Nisin on *S. aureus* was better at high pH; however, the combination of these two compounds was better at lower pH ($P < 0.05$).

The MBC is the best pH value to each bacterium. The antibacterial effects of Nisin and lysozyme and combination of these two compounds showed that these compounds increased antibacterial activity on the growth of *E. coli*, *L. monocytogenes*, *S. aureus*

and *S. typhimurium* in the TSB medium at the temperature of 24°C. There was also a significant difference in the absorption of microorganisms in different pH between control and experimental groups ($P < 0.05$).

The MIC of an antibacterial is defined as the maximum dilution of the product. It will inhibit the growth of a test microorganism and MBC as the lowest concentration of antimicrobial. Furthermore, it will prevent the growth of an organism after subculture on the antibiotic-free media. The MIC and MBC were obtained according to the **Table 1, 2 and 3**.

Table 1. Minimum inhibitory concentrations (µg/Ml; MIC) and minimum bactericidal concentrations (µg/Ml; MBC) for Nisin

Type of bacteria		pH					
		5.5	6	6.5	7	7.5	8
S. typhimurium	MIC[a]	625	1250	312	78	19	19
	MBC[b]	625	2500	625	156	78	39
S. aureus	MIC	312	625	78	156	19	19
	MBC	1250	1250	156	312	78	19
E.coli	MIC	1250	1250	625	78	39	78
	MBC	1250	1250	625	156	78	78
L. monocytogenes	MIC	5000	5000	2500	625	312	625
	MBC	0	0	5000	2500	625	1250

[a] MICs were determined as well concentrations where the average OD from three replicate wells was not 0.05 greater than uninoculated controls.
[b] MBCs were determined as well concentrations where there was at least a 99.9% decrease in CFU ml $^{-1}$.

Table 2. Minimum inhibitory concentrations (µg/Ml; MIC) and minimum bactericidal concentrations (µg/Ml; MBC) for lysozyme

Type of bacteria		pH					
		5.5	6	6.5	7	7.5	8
S. typhimurium	MIC[a]	0	0	0	0	0	0
	MBC[b]	0	0	0	0	0	0
S. aureus	MIC	39	39	0	0	39	39
	MBC	156	312	0	0	312	156
E.coli	MIC	0	0	0	0	0	0
	MBC	0	0	0	0	0	0
L. monocytogenes	MIC	0	0	0	0	0	0
	MBC	0	0	0	0	0	0

[a] MICs were determined as well concentrations where the average OD from three replicate wells was not 0.05 greater than uninoculated controls.
[b] MBCs were determined as well concentrations where there was at least a 99.9% decrease in CFU ml -1 .

Table 3. Minimum inhibitory concentrations (µg/Ml; MIC) and minimum bactericidal concentrations (µg/Ml; MBC) for Nisin + lysozyme

Type of bacteria		pH					
		5.5	6	6.5	7	7.5	8
S. typhimurium	MIC[a]	19	625	625	312	39	39
	MBC[b]	312	1250	625	625	78	78
S. aureus	MIC	19	312	312	156	39	39
	MBC	156	312	312	312	78	78
E.coli	MIC	19	1250	625	156	39	5000
	MBC	156	1250	1250	312	78	5000
L. monocytogenes	MIC	2500	0	5000	2500	5000	39
	MBC	5000	0	5000	5000	5000	78

[a] MICs were determined as well concentrations where the average OD from three replicate wells was not 0.05 greater than uninoculated controls.
[b] MBCs were determined as well concentrations where there was at least a 99.9% decrease in CFU ml -1 .

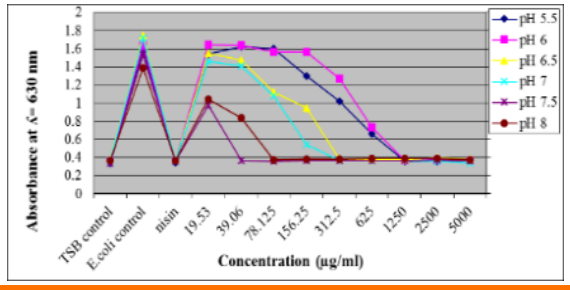

Figure 1. Effect of different Nisin concentrations on *E. Coli*

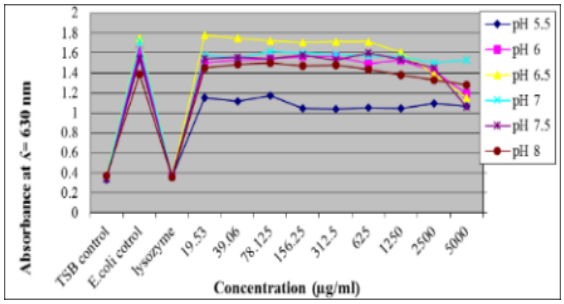

Figure 2. Effect of different lysozymeon E. Coli concentrations

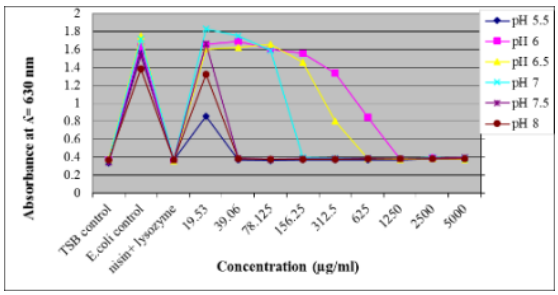

Figure 3. Effect of different concentrations combinations of Nisin and lysozymeon *E. Coli*

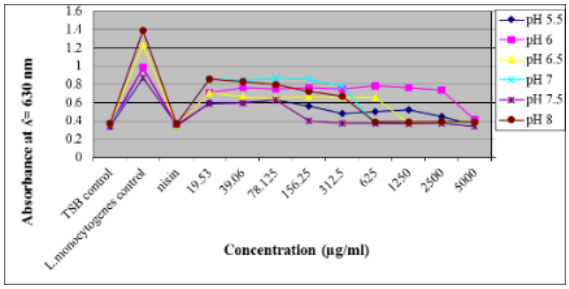

Figure 4. Effect of different Nisin concentrations on *L. Monocytogenes*

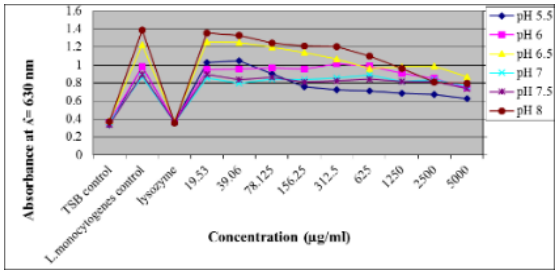

Figure 5. Effect of different concentrations of lysozymeon *L. Monocytogenes*

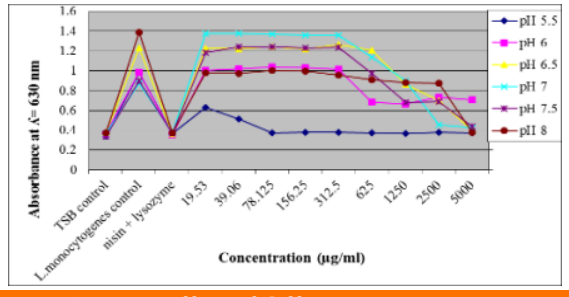

Figure 6. Effect of different concentrations combinations of Nisin and lysozymeon *L. Monocytogenes*

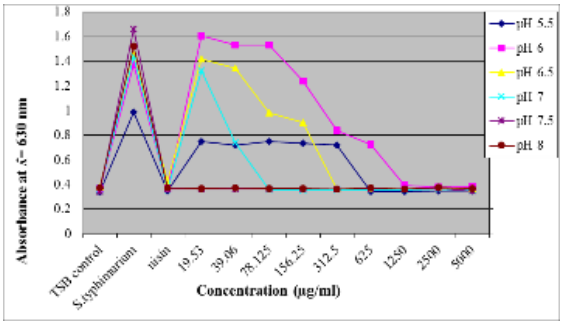

Figure 7. Effect of different Nisin concentrations on *S. Typhimurium*

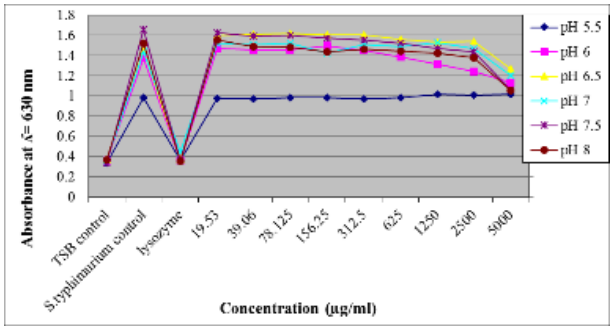

Figure 8. Effect of different concentrations of lysozymeons. *Typhimurium*

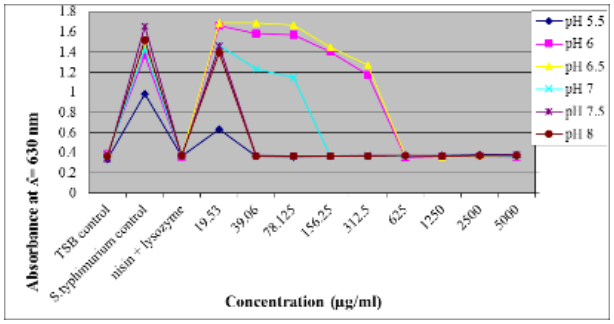

Figure 9. Effect of different concentrations combinations of Nisin and lysozymeon *S. Typhimurium*

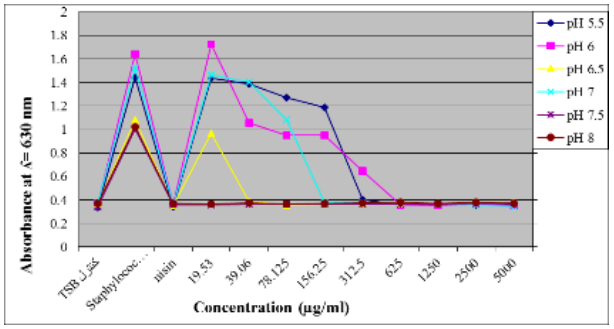

Figure 10. Effect of different Nisin concentrations on *S. Aureus*

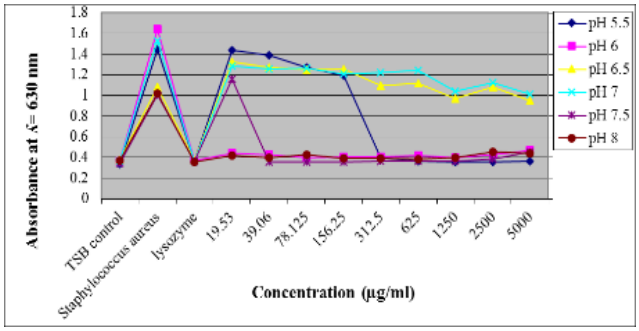

Figure 11. Effect of different concentrations of lysozymeon *S. Aureus*

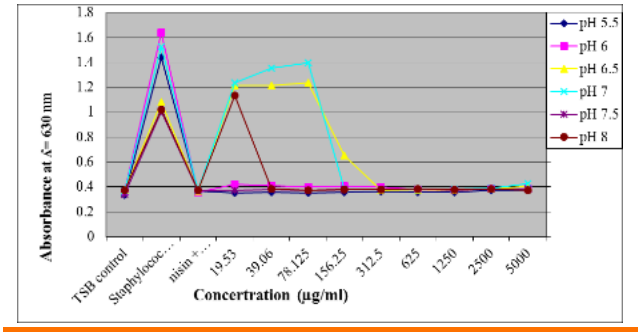

Figure 12. Effect of different concentrations combinations of Nisin and lysozymeon *S. Aureus*

Discussion

In several previous studies, the mixture of Nisin and lysozyme was more potent in its activity than using them separately. Antimicrobial effects of lysozyme, Nisin and Ethylenediaminetetraacetic acid (EDTA) (mix1: 250 ppm) lysozyme, 250 ppm Nisin, 5 Mm EDTA, Mix2: 500 ppm lysozyme, 500 ppm Nisin, 5 EDTA Mn) on bacterial growth in patty of ostrich packed in air, vacuum, and modified atmosphere have examined that a low concentration of lysozyme and Nisin show the best antioxidant effects and high concentrations of lysozyme and niacin show the highest levels of color. Moreover, the undesirable odor of untreated patty was faster than the treated samples (Mastromatteo *et al.*, 2010a), and then antimicrobial synergistic activity of lysozyme (250 ppm), Nisin (250 IU/g), and EDTA (20Mm) against *L .monocytogenes* and bacterial pathogens of meat in patty of ostriches packed in air and vacuum studied.

The results show that antimicrobial treatment, reduced the number of *L. monocytogenes* in ostriches patty to less than the official EU limit (< 2 log CFU/g) (Mastromatteo *et al.*, 2010b).

Furthermore, Sikin et al. showed that application of high pressure nitrous oxide with heat and Nisin and lysozyme have synergistic effects on both vegetative and spore-forming bacteria in milk (Sikin *et al.*, 2017). Park et al. indicated that lysozyme antibacterial activity can be enhanced by attachment to chitosan, which can increase the use of these substances in the food industry (Park *et al.*, 2004). In another study that was conducted on inhibitory effects of Reuterin, Nisin, lysozyme and nitrite inhibition against vegetative cells and spores of dairy-related Clostridium, Reuterin and Nisin were promising candidates for controlling Clostridium growth and preventing late blowing defect in cheese (Ávila *et al.*, 2014). Chai et al. concluded that the combined effect of Nisin and lysozyme leads to inactivation of germinated and outgrowing *Clostridium difficile* spores. Therefore, the findings of these researchers provide the possibility of developing a safe and effective method to inactivate *C. difficile* spore (Chai *et al.*, 2017).

White studied the antibacterial effects of Carvacrol and Fosvitin with Nisin alone or in combination on human intestinal pathogens and based on the results of the studies. It was concluded that using Fosvitin and Carvacrol has a potentially good combination to control the growth of food pathogens in onion and mushroom soup and to produce microbial immunity in foods with potential hazard. The combination of Nisin and Fosvitin is far less effective in preventing the growth of pathogen agents in the soup (White, 2011). Tong et al. evaluated the effect of Nisin and sodium fluoride, or chlorhexidine on *Streptococcus mutans*, and stated that Nisin and sodium fluoride compounds cause more severe damage to *S .mutans*. Furthermore, in antibiofilm Nisin test with sodium fluoride, a more bactericidal effect was observed on *S. mutans* biofilm that can be combined to produce drugs to prevent dental caries (Tong *et al.*, 2011). Govaris et al. investigated the antimicrobial activity of oregano, Nisin and their combination with *Salmonella enteritidis* in processed lamb meat during storage in a refrigerator. It was concluded that the combination of oregano 0.6% with Nisin (500 IU/g) has a stronger antimicrobial effect against *S. enteritidis*, and the inhibitory effect at 10°C is higher than 4°C (Govaris *et al.*, 2010). Silva et al. examined the effect of nanovesicle-encapsulated Nisin on the growth of *L. monocytogenes* in milk. They showed that it can be used to overcome the persistence and reduction of food interactions by combining low temperatures to control *L. monocytogenesis* of milk (Silva *et al.*, 2010). Malinowska-Paczyk Kołodziejska examined the effects of lysozyme and Nisin on some bacteria at high pressure and temperature below zero degrees. Gram-positive and -negative bacteria were tested at 193 MPa and temperature -20 °C in the presence of lysozyme and Nisin at 400 mg/mL concentration. *Pseudomonas fluorescens* were sensitive to high pressure and low temperatures. The growth of *E. coli* decreased; however, the growth of the two strains of *S. aureus* was negligible (Malinowska-Paczyk and Kołodziejska, 2009). Finally, it can be concluded that, the least effect of lysozyme was on *E. coli*.

Conclusions

In this study, the least effect of lysozyme was on *E. coli*. The best performance of Nisin was on *S. aureus* bacteria and the least effect of Nisin was on *L .monocytogenes*. The combined effect of these two substances on *E. coli* was better than each individually, especially in pH 5.7 and 8. However, in the case of *L. monocytogenes*, this effect was less common. *S. typhimurium* was better at pH 5.5 and 6; while in other pH, Nisin was better. *S .aureus* works better at all pH except 6.5 and 8. In the case of *E. coli*, these two substances were more effective in all pH except pH 7. Therefore, combined use of Nisin and lysozyme against Gram-positive bacteria show co-operation and the mechanism of killing the bacteria is strengthened. These two substances had the most combined effect of on *S. aureus*. Thus, it is suggested to food producers that combination of Nisin and lysozyme can be used as an effective preservative in food.

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Authors' Contribution

All authors contributed equally in designing, performing experiments, analyzing the data and writing the manuscript.

Conflict of Interest

There is no conflict of interest and this study has not been sent elsewhere.

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