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* Corresponding author:
rashidi.azade@yahoo.com
Department of Food Hygiene and Quality Control, School of Veterinary Medicine, Shahrekord University, Shahrekord, Iran.

Postal code: 8915173160
Tel: +98 353-38209100

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 concentration (MBC) of these combinations on the bacteria of Escherichia coli, Staphylococcus aureus, Salmonella typhimurium 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-acetylglicosamine 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

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Nisin were zero, 19.53, 39.69, 13.78, 25/156, 312, 625, 1250, 2500, 5000 μg/mL. The dilution method was adopted from 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 Richard, 2003). Lysozyme and Nisin stock solutions at 10000 μg/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 10⁵ 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 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 | 625 | 1250 | 312 | 78 | 19  | 19 |
|                  | MBC | 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|

* MICs were determined as well concentrations where the average OD from three replicate wells was not 0.05 greater than uninoculated controls.
* 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 | 0   | 0  | 0   | 0  | 0   | 0  |
|                  | MBC | 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  |

* MICs were determined as well concentrations where the average OD from three replicate wells was not 0.05 greater than uninoculated controls.
* 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 | 19   | 625 | 625 | 312 | 39  | 39  |
|                  | MBC | 312  | 1250| 625 | 625 | 78  | 78  |
| S. aureus        | MIC | 19   | 312 | 312 | 156 | 39  | 39  |
|                  | MBC | 156  | 1250| 625 | 625 | 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.
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., 2014). 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. monocytogenes of milk (Silva et al., 2010). Malinowska-Paczyk and 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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