Synergism Effect of Nisin Peptide in Reducing Chemical Preservatives in Food Industry

Hamed Haddad Kashani[1], Hosein Nikzad[2], Saed Mobaseri[*3], Elahe Seyed Hoseini[4]

Anatomical Sciences Research Center, Kashan University of Medical Sciences, Kashan, Iran[1].
Anatomical Sciences Research Center, Kashan University of Medical Sciences, Kashan, Iran[2].
Department of Microbiology, Science Faculty, Azad University, Qom, Iran[3].
Research Center for Sciences and Biotechnology, Lavizan, Tehran. Iran[4]

Abstract: Due to increasing interest to natural preservatives, many studies have been performed in recent years. Nisin peptide as a natural preservative is very interesting for the control of food pathogens and microorganisms of food spoilage. In this study antibacterial activity of nisin and its synergism in decreasing concentrations of common chemical preservatives like sodium nitrite and benzoic acid against standard bacteria of *Staphylococcus aureus* and *Lysteria monocytogenes* has been investigated. For this purpose, the MIC (minimum inhibitory concentration) of nisin peptide, sodium nitrite and benzoic acid were evaluated at different pH based on the dilution method. Results showed that these compounds have antibacterial activity against *Staphylococcus aureus* and *Lysteria monocytogenes*. MIC for nisin peptide, sodium nitrite and benzoic acid in *Staphylococcus aureus*, was 350, 200, 25 (ppm) respectively. These values were 100, 200, 10 (ppm) in *Lysteria monocytogenes.* It has been also shown that simultaneous use of nisin with chemical preservatives sodium nitrite and benzoic acid reduced MIC of these compounds against two strains of bacteria. This synergistic effect of nisin could reduce the use of chemical preservatives in food industry.

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Introduction:

In food industry achieving a healthy product with a proper maintenance can be one of the main indexes in order to food producing. Therefore variety of methods has been evaluated. Nowadays, preservatives are among the methods that used commonly in food processing. To prevent spoiling of food that created by microbial growth or chemical alterations, preservatives that either natural or artificial, are added to food products. Dietary antimicrobial preservatives are one of the most important groups of conservative that improve food quality, extend the shelf life, waste reduction and processing cost. In recent years, nitrate and sodium nitrite as a chemical preservatives have been widely used in meat and dairy processing with a suitable antimicrobial effects. (Goldstein 1). But it should be noted that application of these compounds in 1960 following liver toxicity of domestic animals fed canned fish meal content large amounts of sodium nitrite was confronted with challenges. Studies have shown these compounds in gastrointestinal tract are converted to carcinogenic compound called nitrous amine, which is effective in appearance of malignant tumors. The presence of nitrates in food may cause Met-hemoglobin phenomenon, created by oxidation of Oxy-hemoglobin to Ferry-hemoglobin. This can be fatal, especially in newborn infants, since reduced met-hemoglobin has low oxygen-carrying capacity that may eventually lead to anemia and Blue Baby Syndrome. Other adverse effects of these compounds are its deterrent effect on nutrient absorption at intestine (Fan & Steinberg 2).

Benzoic acid and its salts (sodium, calcium, potassium) are used such other chemicals in food industry in order to keep jam, initiators, beverages, salads and olives. There are also concerns about the use of benzoic acid and its derivatives including reaction possibility of these compounds with ascorbic acid (vitamin C) and formation of small amounts of benzene, which is a carcinogenic substance. Professor Peter Piper of the University of Sheffield claims that sodium benzoate by itself can damage and inactivate vital parts of DNA in a cell's mitochondria. Mitochondria consume oxygen to generate ATP, the body's energy currency. If they are damaged due to disease, the cell malfunctions and may enter apoptosis. Research published in 2007 for the UK's Food Standards Agency (FSA) suggests that certain artificial colours, when paired with sodium benzoate may be linked to Attentio Deficit-Hyperactivity Disorder (ADHD).(McCann D. 3). The results of this study indicate that certain combination of artificial food colors and sodium benzoate preservatives is associated with increasing hyperactivity behavior in children. Therefore the adverse effects of chemical substances in food preservation considering to natural and biological preservatives with inhibitory effects on wide spectrum of pathogenic microorganisms, which having no side effects and improve the smell and taste of food, have expanded in recent years. Accordingly, Hurdle technology as a new method for producing safe, sustainable, nutritious, tasty and economical food is developed. Combination use of natural preservatives such as herbal essential oils and probiotic agents are important in order to achieve a high level of health, hygiene and shelf life of food products. According to Hurdle technology, more than 60 affordable and safe options are presented to improve health and quality of foods that use of bacteriocin as probiotic products is one of the options. Maximum allowable concentration of nitrites in meat products is 200 ppm. European Union in 1985 announced 50mg/lit permitted level of Nitrites in drinking water (Ivanov 4).

In 1969, nisin peptide was approved by F.A.O & W.H.O (Food and Agriculture Organization / World Health Organization) to use as a preservative in food issues. (Noonpakdee et al 5). Nisin peptide as a bacteriocin agent has been operating since 1987 as a formal additive in food and dairy products. This peptide is composed of 34 amino acids, which produced by the dairy starter culture *Lactococcus lactis* subsp. *lactis*. (Liu, W., N. Hansen 6). Nisin was not toxic and is rapidly inactivated by digestive enzymes. Today's, this bacteriocin has a relatively broad spectrum of inhibitory activity against gram-positive bacteria and is commonly used as a preservative in many countries around the world in foods such as canned goods, dairy products, pasteurized cheeses, dairy desserts, , frozen meats and sea foods. (Hurst, A, 7, Ross 8).

Due to complications of chemical preservatives like sodium nitrite and benzoic acid compounds, the objective of this research was to evaluate effect of nicin bacteriocin as a natural and harmless preservative, and its capability on MIC and MBC reduction of the chemical preservatives.

Materials and Methods:

1- Solution preparation of chemical preservatives ;(sodium nitrite, Benzoic acid) and nisin peptide: Suitable solvents for Benzoic acid and sodium nitrite (Merck) are 90% ethanol and distilled water respectively. In case of nisin peptide, 2% HCL (High Media) are applied**.** Benzoic acid and sodium nitrite stock solution equal to 30mg/ml combined with 10mg/ml nisin peptide was prepared and each of solution was sterilized separately by millipore filter with a diameter of 0.22 micrometers.

2- Microbial strains: Strains of *Staphylococcus aureus ATCC 1112* and *Lysteria Monocytogenes ATCC 1301* as standard samples used in experiments**.** After confirming above strains according to laboratory criteria, primary cultures of bacteria were prepared with TPB (Triptose phosphate broth) medium at proper temperature of 37 C °.

3- MIC and MBC tests: at this study MIC and MBC experiments were done based on macro dilution in *Mueller Hinton Broth* medium with initial bacteria amount of ~ 5×10^5 CFU / ml (prepared based on 0.5 Mac Farland) were conducted according to NCCLS protocol in two stages.

In the first stage MIC and MBC of sodium nitrite, Benzoic acid and nisin compounds separately at three different pH for each strain were evaluated to determine the best effectiveness of compounds at the optimum pH. So culture medium tubes containing *Mueller Hinton Broth* (MHB) individually prepared with pH of 7, 6 and 5.5 in association of bacterial suspension and dilution series of preservatives with concentrations of 4500, 3500, 2500, 1500, 750, 500, 350, 200, 100, 50, 25, 10 (ppm) were made and the lowest concentration that inhibits bacterial growth was detected. MBC evaluations were carried out in *Mueller Hinton Agar* (MHA) medium based on the lowest concentration that inhibits bacterial growth rate up to 99.9%.

In the second stage, MIC and MBC of sodium nitrite, Benzoic acid in combination of nisin peptide were evaluated at the optimum pH for each strain. accordingly in a separate tubes containing MHB medium and bacterial suspension, dilution series of benzoic acid and sodium nitrite with a concentration of 5, 25, 50, 100, 200, 350, 500 (ppm) were prepared and then to the sample tubes, nisin peptide was added equal to its MIC concentration at optimum pH. Tubes were incubated for 18 hours at 37C° and the experiments were repeated three times.

Results:

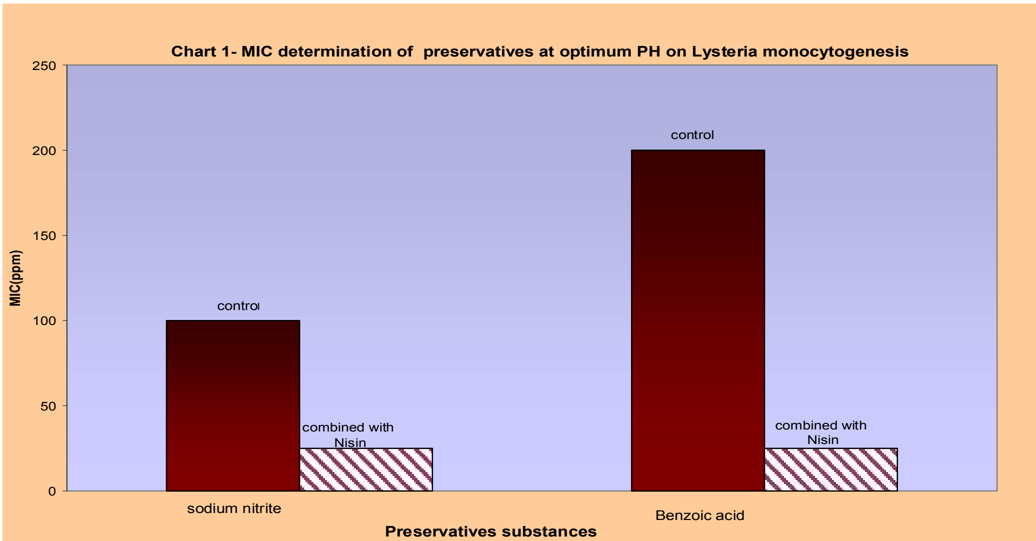
First stage results of experiments showed sodium nitrite MIC for *S.aureus* at 7, 6 and 5.5 pH was 4500, 750 and 350 ppm respectively and these values in *L.monocytogenes* were 3500, 750 and 100 ppm respectively.

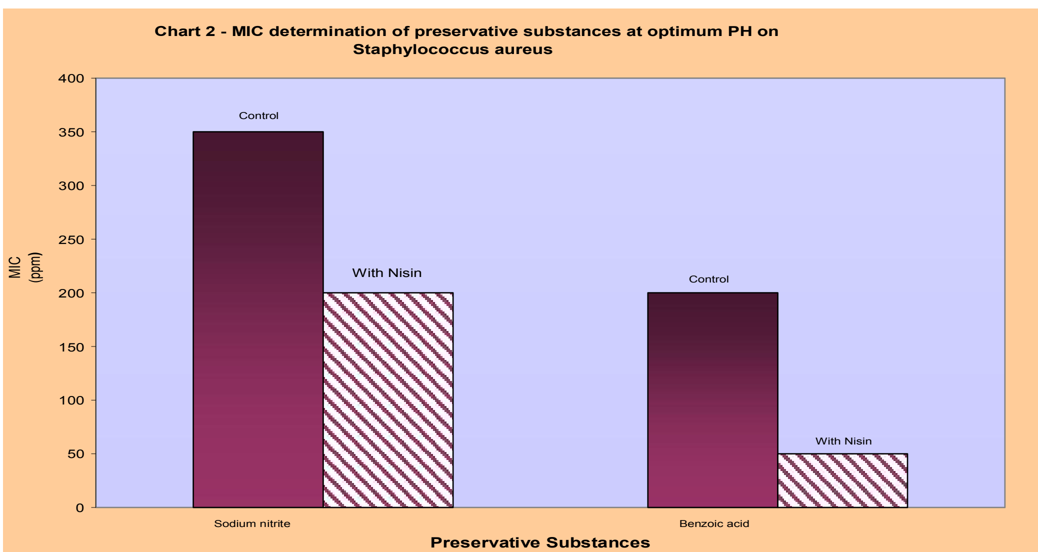
Benzoic acid MIC in S.aureus at 7, 6 and 5.5 pH was 750, 500 and 200 ppm respectively, and these values for *L.monocytogenes* were 750, 500 and 200 ppm. MIC of Nisin also at 7, 6 and 5.5 pH in *S.aureus* and *L.monocytogenes* was 100, 50, 25 ppm and 50, 25, 10 respectively.

Therefore, the results can be concluded that optimum pH of three compositions for highest activity and lowest effective concentration of MIC and MBC are equal to 5.5.

The second stage of experiment, at the optimum pH when chemical preservatives, sodium nitrite and benzoic acid, were applied in association of nisin peptide showed the MIC of compounds against two strains of bacteria were reduced significantly so that sodium nitrite and benzoic acid MIC is reduced to 200, 50 ppm respectively for *Staphylococcus aureus* and these values in *Lysteria Monocytogenes* were 25, 25 ppm. (Figure1, 2).

Therefore, according to results, presence of nisin as a safe preservative has reduced significantly MIC and MBC of chemical preservatives like sodium nitrite and Benzoic acid.





Discussion:

The effects of preservatives on microorganisms are carried out with different mechanisms. Nitrite in acidic pH can be converted to nitrous acid, and has been shown this compound is capable of reaction with various materials, such as myoglobins, ascorbic acid, phenols, secondary structure of amines, compounds with amine agent groups, metalloporphyrins and iron-sulfur cluster, and inhibits the growth of microorganisms. (Ferric C. Fang 9). Benzoic acid also at low pH has inhibitory activity against microorganisms, in this condition due to increased membrane permeability, uncatalyzed form of compound can pass freely through the cytoplasmic membrane and following the cell influence shows its effect. (Booth & Kroll 10).

Nisin peptide with bacteriocin activity has a relatively broad spectrum of inhibitory activity against gram-positive bacteria and bacterial spores. (Blackburn et. al, P 11). This peptide binds to specific protein receptor on the cell wall and destabilizes the cell membrane function so increases permeability of the cytoplasmic membrane to ions, especially cations. Producer strains and many resistant gram-positive bacteria adsorb nisin to the cell wall with no consequences. These protein receptors may also function as receptors for other metabolites which are transported into the cell. The release of important cations, such as K+, from nisin-treated cells results in a dissipation of the proton motive force potential. This force serves to control many membrane functions such as the active transport of amino acids and sugars across the cellular membrane. These and other cell membrane functions that depend on the existence of the proton motive force are lost following nisin peptide treatment. A further consequence is losses of cellular ATP reserves are expended in an attempt to maintain the membrane potential. The decrease in the intracellular concentration of ATP inhibits many-energy dependent reactions in the cells. (Hurst and Hoover, 12). Finally, this process reduces the pH equilibrium of cells and collapses the proton motive force (PMF), so that eventually suppress all biosynthetic processes. (Chen & Hoover 13).

In this research, synergism and effect of simultaneous use of nisin peptide with chemical preservatives of sodium nitrite and benzoic acid and MIC reduction of these compounds were studied. Results showed nisin could reduce significantly sodium nitrite MIC at the studied bacteria so that the amount of nitrite was reduced to below 200 ppm, which is the limit of nitrite consumption. These studies also showed nisin could reduce Benzoic acid MIC in *Staphylococcus aureus* and *Lysteria monocytogenes* from 200ppm to below 50ppm. The allowable amounts of nitrate are 120 to 200ppm, but rarely may exceed this amount from 150ppm. Food and Drug Administration of America (F.D.A) pointed out the amount of nitrite and nitrate that should not be more than 200ppm and 500 ppm respectively (Federal Register 14). In general, to prevent the activity of pathogenic bacteria, 80 to 150ppm of the compounds is necessary. Benzoic acid and its sodium salt derivatives in concentration of 50-500ppm inhibit activity and growth of poisoning and pathogenic bacteria. There are many reports about combined effect of preservatives and nisin peptide on various microorganisms. For example, Karim et al (2010) reported that the combination of nisin peptide in association of chemical inhibitors such as EDTA, citrate and phosphate may be more effective against *Escherichia coli* and *Salmonella*. (15). Chung & Huncock also showed the effects of lysozyme with nisin could be more effective against *Lactic bacteria*. Synergism effects of nisin in association of Monolaurin and lauric acid have also observed against *Streptococcus agalactic* and *Lactobacillus plantarum* respectively.Chung & Huncock and in 2000 showed there are synergism between Lysozyme and Nisin against *Lactic bacteria* (Chung & Hancock 16) Synergism effects of nisin in association of Monolaurin and lauric acid have also observed against *Streptococcus agalactic* and *Lactobacillus plantarum* respectively. (Stefania & Loredana 17). Alexander O. Gill, Richard A. Holley (2002) observed the combination of EDTA and Nitrite can effect on *E.coli* and *Salmonella* (18, 19). Rayman and colleagues(20) also in 2006 showed combination of 40ppm nitrite with 75-100ppm nisin peptide can inhibits the growth of bacteria such as *Clostridium sporogenes*, confirming the results of synergism between nisin and nitrite combination. It is clear that the low level of nitrite with the least adverse effect is sufficient for meat maintenance. This study has also shown sodium nitrite and Benzoic acid combined with nisin peptide has synergism effect, so that in *Staphylococcus aureus*, active concentrations of sodium nitrite and benzoic acid based on MIC, have been reduced 40 and 75 percent respectively. Also concentrations of sodium nitrite and benzoic acid in Lysteria Monocytogenes were significantly reduced to 75 and 87 percent. Reduction of minimum effective concentrations of chemical compounds in association of nisin peptide is due to permeability of cell membrane by function of nisin peptide, so that this peptide penetrates and intruded into the lipopolysaccharide of cell membrane causing instability in biophysical connections, then by generating pores, increases cell permeability of compounds such as sodium (Chung, K. T, et. al, 21) nitrite and benzoic acid, which results enhanced sensitivity of bacteria to these compounds and thus reduces minimum active concentrations against two experimented strains. Mechanism modality of nitrite effect on bacteria are still not entirely clear, but is thought that, next to the production of nitrous amine, which was discussed, nitrite may impact on sulfhydryl compounds of microorganisms causes changing in their metabolism and eventually disrupt the bacterial growth and proliferation. Antimicrobial effect of nitrite is depended on pH of products, so by falling of pH equal to one unit, uncatalyzed part of nitro acid (HNO2), which is operating as an antimicrobial agent, increases its effect up to 10 fold, and therefore is recommended to add small amount of edible acids to products intended to reduce level of pH. (Foegeding, P. M. et. al, 22) Antimicrobial and anti bacterial activity of Benzoic acid also has a direct relation with pH, so that at the low pH shows more potency. Accordingly, it was also observed that at three investigated pH, MIC and MBC of chemical preservatives, sodium nitrite and benzoic acid, had minimum active concentration in the lowest pH, which indicate the influence of pH on maximum antimicrobial activity. Functionally Benzoic acid interferes in cellular energy transferring system and inhibits activity of enzymes that associate in metabolism of acetic acid and Krebs cycle, so that after uptake of acid by cells, intracellular pH may come down to 5 or lesser and anaerobic fermentation of glucose by Phosphofructokinase is reduced up to 95%, which leads to bacterial disruption. (Kristo E, 23, Parmar & Forestry 24, Jin et al 25).

As mentioned, nisin peptide next to the increasing of bacterial cell membrane permeability, disrupt proton pumps in the membrane structure that results in disturbance of proton gradient and intracellular pH of bacteria. (Klaenhammer, T. K.26). Therefore besides increasing in membrane permeability, pH changes also cause to increase activity and ability of compounds such as sodium nitrite and benzoic acid, which result to reduce the minimum active concentration.

Based on the findings obtained in laboratory conditions, other factors also may be effective on the MIC and MBC of preservatives, so it is necessary to assess the results at industrial conditions.

*** Corresponding Author:**
Saed Mobaseri, Department of Microbiology, Science Faculty, Azad University, Qom, Iran.
Email: saed_mob@yahoo.com

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