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ORIGINAL RESEARCH PAPER

Evaluation of antibacterial effect of magnesium oxide nanoparticles with nisin and heat in milk

*M. Mirhosseini**

Department of Biology, Payame Noor University, Tehran, Iran

ABSTRACT

***Objective(s)*:** The objective of this study was to investigate the antibacterial activities of magnesium oxide nanoparticles (MgO NP) alone or in combination with other antimicrobials (nisin and heat) against *Escherichia coli* and *Staphylococcus aureus* in milk.

Materials and Methods: First, the combined effect of nisin and MgO nanoparticles was investigated in milk. Then the combined effect of nisin, heat and MgO nanoparticles was assessed in milk. Also the scanning electron microscopy was used to characterize the morphological changes of *S. aureus* before and after antimicrobial treatments.

***Results*:** The results showed that MgO NP have strong bactericidal activity against the pathogens. A synergistic effect of MgO in combination with nisin and heat was observed as well. Scanning electron microscopy was revealed that MgO NP treatments in combination with nisin distort and damage the cell membrane, resulting in a leakage of intracellular contents and eventually the death of bacterial cells.

***Conclusion*:** These results suggested that MgO NP alone or in combination with nisin could potentially be used as an effective antibacterial agent to enhance food safety.

Keywords: *Antibacterial activity, Foodborne pathogens, Magnesium oxide, Nanoparticles, Nisin*

INTRODUCTION

Outbreaks caused by foodborne pathogens continue to draw public attention to improve food safety. It is estimated that approximately each year roughly 48 million Americans people get sick, 127,839 are hospitalized, and 3,037 die of foodborne diseases in the United States [1]. Therefore, in order to solve this problem, it is highly necessary to develop effective antimicrobial agents for improvement in food safety and shelf-life. In recent years, the application of inorganic antimicrobial agents has attracted much attention for the control of pathogenic microorganisms [2-3].The specific advantages of inorganic antimicrobial compounds are the improved safety and stability under high temperature treatments [4-7].

Inorganic nano metal oxide such as ZnO, MgO and CaO have been investigated as antimicrobial agents [8-11]. MgO is an important inorganic oxide with typical wide band-gap [12]. It has been used in many applications such as catalysis, catalyst supports, toxic waste remediation, refractory materials and adsorbents, additive in heavy fuel oils, reflecting and anti-reflecting coatings, superconducting and ferroelectric thin films as the substrate, superconductors and lithium ion batteries, etc [13-14]. In medicine, MgO is used for the relief of heartburn, sore stomach, and acid indigestion, as an antacid, detoxifying agent, and for bone regeneration [15-16]. Recently, MgO nanoparticles applied in cancer therapy such as nano-cryosurgery and hyperthermia [17]. MgO nanoparticles also have considerable potential as an antibacterial agent [18-19]. Nisin is ribosomal synthesized peptides with antimicrobial activity. Also nisin is being presently widely used as a preservative in several food products such as processed cheese, dairy desserts, and canned foods. This antimicrobial protein exhibits inhibitory activity against Gram-positive bacteria including spore-

*Corresponding Author Email: *m.mirhossaini@gmail.com*
Tel: (+98) *913-3730895 (P.O. BOX 119395-3697)*

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forming bacteria and other spoilage and pathogenic bacteria [20]. However, few studies have been reported on the use of nisin in combination with MgO NP.

The objective of this study was to investigate the antibacterial activities of MgO NP and its synergistic effect in combination with nisin and heat against foodborne pathogens (*E. coli* and *S. aureus*).

MATERIALS AND METHODS

Materials

MgO nanoparticles with a diameter of 20-30 nm from US Nano Company with purity% 98/99 and Nisin from Sigma Company USA were prepared.

Bacterial strains and culture conditions

The following bacterial strains were used in this study: *E. coli* PTCC1330, and *S. aureus* PTCC1112. These mentioned bacteria were obtained from the culture collection of the I.R. Department. Moreover, Stock cultures were maintained at -80 °C. The strains were propagated on Tryptic Soy Agar (TSA; Merck, Darmstadt, Germany) at 37 °C and maintained at 0– 2 °C before use. Each strain was cultured in Tryptic Soy Broth (TSB; Merck, Darmstadt, Germany) at 37 °C for 24 h, harvested by centrifugation at 4000 g for 20 min at 4 °C and washed three times with buffered peptone water. The final pellet was resuspended in buffered peptone water, corresponding to approximately 10^7–10^8 CFU ml^{-1} and mixed cocktails prepared by blending together equal volumes of each test strain.

Antibacterial activity of the combination of MgO nanoparticles and Nisin

The milks contained (0, 2, 4) mg/ml MgO nanoparticle, (0, 0.008, 0.01) mg/ml Nisin and (2 + 0.008, 4 + 0.008, 2 + 0.01, 4 + 0.01) mg/ml MgO nanoparticle and Nisin were prepared, respectively. Milk samples were then inoculated with the prepared mixed culture cocktails (10^6–10^7 CFU ml^{-1} of each strain). The milk samples were stored at room temperature and examined at 3, 6, 8 and 24 h, respectively, to allow for the recovery and then enumeration of injured cells. All experiments were repeated three times [21].

Aliquots (1 ml) of the treated milk samples were dispersed in 9 ml of 0.2% (w/v) sterile peptone water and then serially diluted (10^{-1}–10^{-5}) in 0.1% sterile peptone water. Manitol salt agar (MSA, Merck, Darmstadt, Germany) was used for isolation and enumeration of *S. aureus.* Eosin methylene blue (EMB, Merck, Darmstadt, Germany) agar was employed for isolation and identification of *E. coli*. Microscopic examination of isolates was performed by staining smears according to the Gram method. Identification and characterization of bacterial isolates up to species level was implemented using various biochemical and sugar fermentation tests [22-23].

Antibacterial activity of the combination of MgO nanoparticles, Nisin and Heat

The milks contained (0, 2) mg/ml MgO nanoparticle, (0, 0.008, 0.01) mg/ml Nisin and (2 + 0.008, 2 + 0.01) mg/ml MgO nanoparticle and Nisin were prepared, respectively. Samples were then inoculated with the prepared mixed culture cocktails (10^6–10^7 CFU ml^{-1} of each strain). Heat treatments were performed at 100 °C in a water bath, with the temperature monitored by inserting a thermometer into a bottle of milk. During heat treatment, sample bottles were vigorously agitated to facilitate uniform distribution of the inoculums. Once the temperature of the sample reached the target treatment temperature of 50 °C and 60 °C the glass bottles were removed and immediately cooled in crushed ice. The samples were stored at room temperature and examined at 3, 6, 8 and 24 h, respectively, to allow for the recovery and then enumeration of injured cells [21].

Examination of cell morphology by scanning electron microscopy (SEM)

S. aureus cultures in the mid-log phase of growth were treated with 4 mg mL^{-1} MgO NP, 0.01 mg mL^{-1} nisin, or 4 mg mL^{-1} MgO NP and 0.01 mg mL^{-1} nisin for 18 h. Aliquots of 1 mL treated and untreated cell suspensions were centrifuged at 10,000 rpm for 5 min and the cell pellets were resuspended in 100 µL TSB. Twenty microliter of cell suspensions were deposited on each glass coverslip. After air drying for 45 min, the coverslips were fixed with a primary fixative solution containing 2.5% glutaraldehyde and 0.1 M imidazole buffer solution (pH 7.2) for 2 h. Subsequently, the fixative solution was exchanged with 0.1 M imidazole buffer, followed by dehydration with a series of ethanol solutions (50, 80 and 100%) with three changes at each concentration. The coverslips were mounted on SEM stubs with carbon adhesive tabs, then sputter-coated with a thin layer of gold using a Sputter Coater. Digital images of the treated and untreated *S. aureus* cells were acquired using a scanning electron microscope (Phenom ProX, Holland) at an accelerating voltage of 10 kV and instrumental magnifications of 35000 [18].

Statistical analysis

Antimicrobial experiments were conducted in triplicate. Data points were expressed as the mean ± standard deviation. Data were analyzed using analysis of variance from SPSS version 16 software. Duncan's multiple range tests were used to determine the significant difference of mean values. Unless stated otherwise, significance was expressed at 5% level.

RESULTS AND DISCUSSION

Antibacterial activity of the combination of MgO nanoparticles and Nisin

Fig. 1 presents the survival of *E. coli* after treatments with MgO NP, nisin and their combinations. Based on the cell reductions at 8 and 24 h, the eight treatments could be divided to tree groups: the treatments in group I were not effective (0.008 and 0.01 mg mL^{-1} nisin); the treatments in group II were less effective and reduced 3.56 log CFU mL^{-1} cells (2 mg/ml MgO nanoparticle), and those in group III were most effective and had increased inhibitory effect (4, 2+ 0.008, 2+ 0.01, 4 + 0.008, 4 + 0.01) mg/ml MgO nanoparticle and nisin. These results demonstrated that nisin alone in group I or MgO alone had either no effect or less effect on bacteria reduction. However, the combination of these two groups decreased the viable cell counts. Apparently, the synergistic antibacterial effects existed and were significant. Fig. 2 shows the survival of *S. aureus* treated by MgO NP, nisin, or their combinations. Similar to *E. coli*, the effect of anti- Staphylococci was dependent on the concentrations of MgO NP, and adding nisin to MgO significantly increased the antimicrobial efficacy of MgO against Staphylococci.

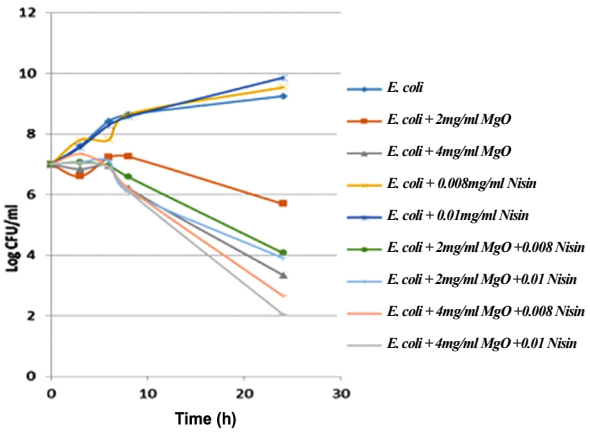

Fig.1. Effects of varying concentrations of MgO NP and nisin on the viability of *E. coli* at high inoculum levels in milk at 25 ºC

Antibacterial activity of the combination of MgO nanoparticles, Nisin and Heat

Fig. 3 shows the survival of *E. coli* exposed to MgO NP, nisin, heat or their combinations. The antibacterial effects of MgO NP were similar to those in Fig. 2. Heat was not reduced the growth of *E. coli* as compared to the controls at 24 h, but its antimicrobial activity was not effective as nisin corresponding to the same concentrations used. Moreover, like nisin, the addition of heat to MgO NP had further enhance the overall antimicrobial activity. Fig. 4 shows the survival of *S. aureus* treated by MgO NP, nisin, heat or their combinations. Similar to *E. coli*, the effect of anti-Staphylococci was dependent on the temperature, and adding heat to MgO NP and nisin significantly increased the antimicrobial efficacy of MgO and nisin against Staphylococci. In addition, Staphylococci cells were more susceptible to MgO NP and nisin treatments than *E. coli* as it was observed that the MgO NP, heat and nisin treatment at the same concentrations achieved greater reduction of Staphylococci than *E. coli* at 8 and 24 h.

Morphological analysis of S. aureus

Morphological observation on the (nisin, MgO NP and MgO NP and nisin combination) treated *S. aureus* using EM analysis showed that the bacteria became more irregular and inhomogeneous in shape (Fig.5 b, c, d) in contrast to control cells which showed a regular, smooth surface with spherical grape-like clusters (Fig. 5a). Most of the cells treated with MgO NP alone, were destroyed as indicated by the abnormal, distorted shape although some of the MgO NP-treated cells developed the formation of irregular and inhomogeneous in shape. However, bacterial lysis was observed and cell surfaces appeared to be altered with ruptured membranes and abnormal morphologies in the combined-treated cells (Fig. 5d). The hurdle technology concept in food processing, sensory quality must also be considered when determining the appropriate microbial intervention strategies [24]. However, some hurdles influence sensory qualities of products, such as color, flavor and texture. In this study, heat, nisin and MgO NP treatments were synergistic and effective in reducing the levels of *E. coli* and *S. aureus* in milke. Heat temperature appeared to be a more critical factor for killing the food-borne pathogens tested here and maintaining them at reduced levels than MgO NP and nisin treatment.

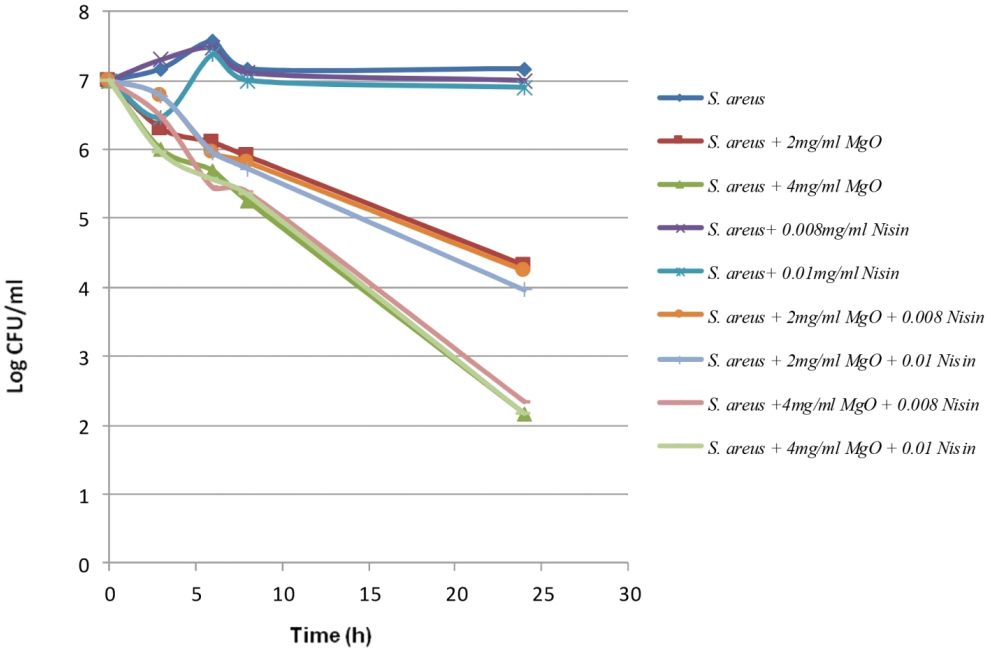

Fig. 2. Effects of varying concentrations of MgO NP and nisin on the viability of *S. aureus* at high inoculum levels in milk at 25 ºC.

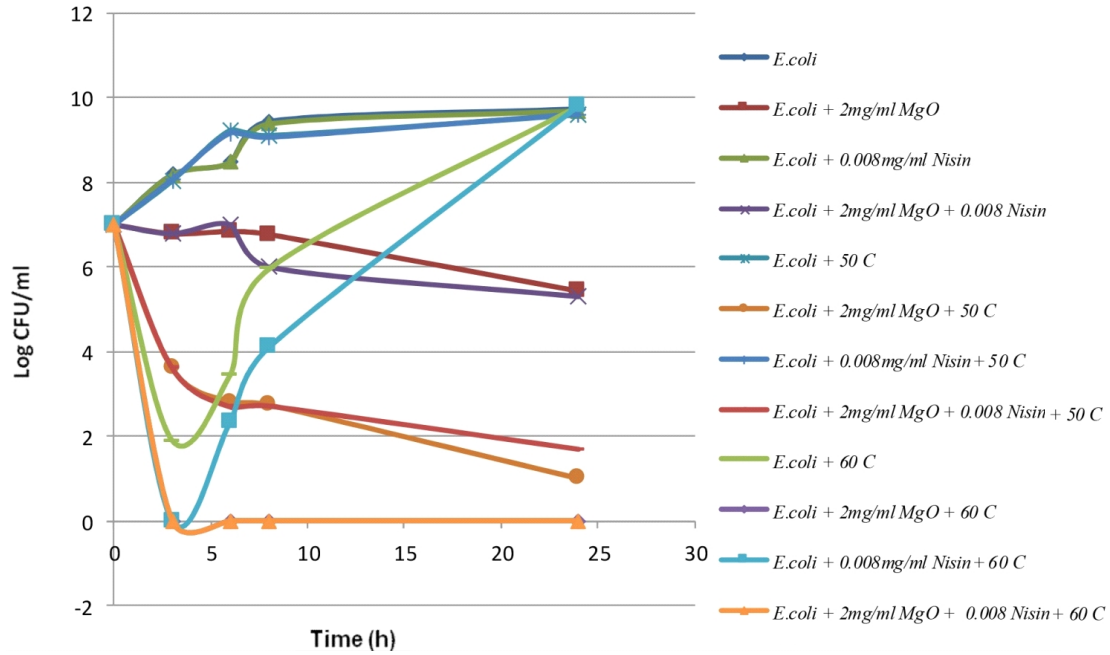

Fig. 3. Effects of varying concentrations of MgO NP, heat and nisin on the viability of *E. coli* at high inoculum levels in milk at 25 ºC.

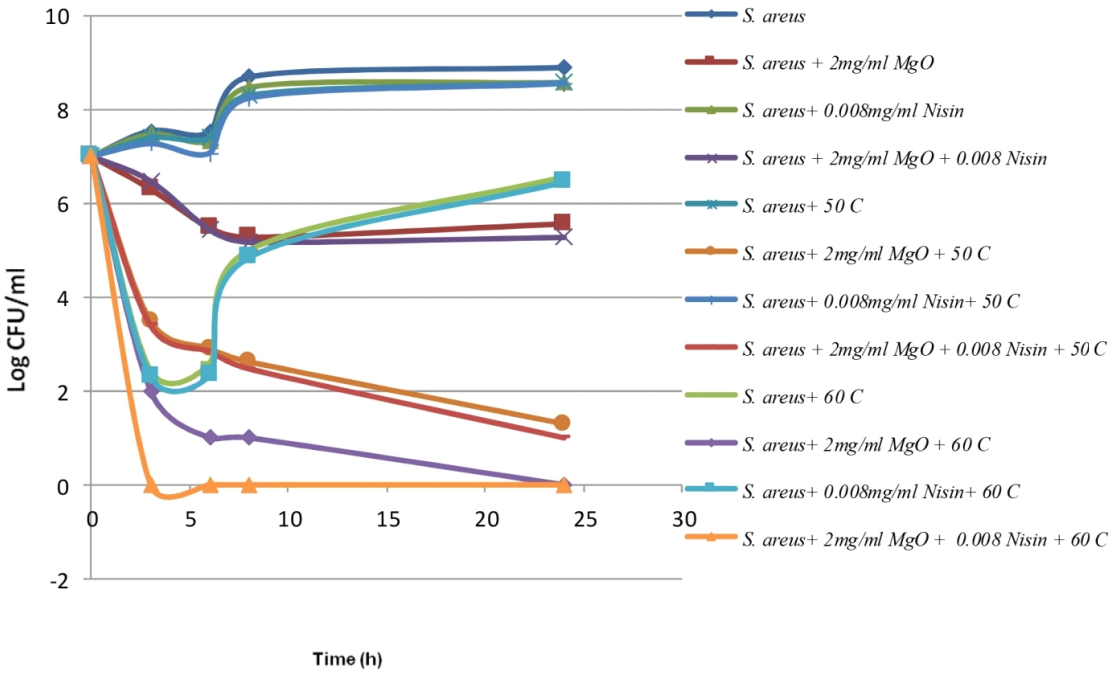

Fig. 4. Effects of varying concentrations of MgO NP, heat and nisin on the viability of *S. aureus* at high inoculum levels in milk at 25 ºC

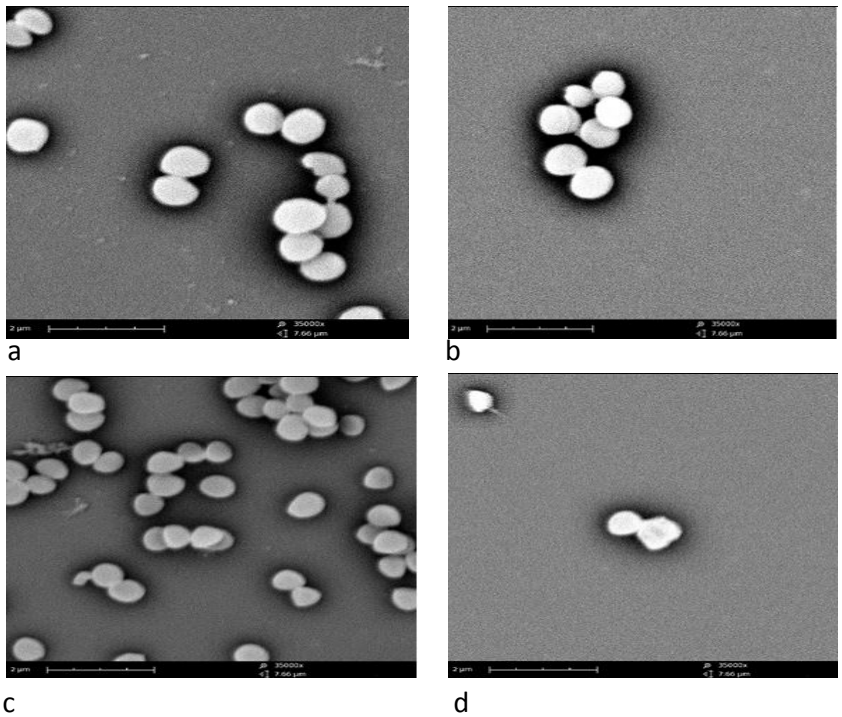

Fig. 5. Scanning electron micrographs of *S. aureus* following treatments with MgO NP and nisin. Bacteria were incubated with a: TSB alone (control), b: 0.01 nisin mg mL^{-1}, c: 4 mg mL^{-1} MgO NP, or d:4 mg mL^{-1} MgO NP / 0.01 mg mL^{-1} nisin, as described under materials and methods

These results indicated that milder heat treatments than those that are commercially employed now could be used to ensure microbial safety, with the potential benefit of being able to produce a product with more "fresh like" sensory qualities.

Jin and He investigated antibacterial activities of magnesium oxide nanoparticles (MgO NP) alone or in combination with other antimicrobials (nisin and ZnO NP) against *Escherichia coli* O157:H7 and *Salmonella Stanley*. The results show that MgO NP have strong bactericidal activity. A synergistic effect of MgO in combination with nisin was observed as well. However, the addition of ZnO NP to MgO NP did not enhance the antibacterial activity of MgO against both pathogens [18].

The SEM images obtained in this work clearly show that the presence MgO NP / nisin leads to significant changes in cell morphology and membrane integrity (Fig. 5). Viability studies showed that the MgO NP / nisin - treated *S. areus* cells were no longer culturable, suggesting disruption of bacterial membrane structure or integrity could be the cause of cell death.

According to currently available information, nanomaterials used in food application include both inorganic and organic substance. Engineered nanomaterials (ENMs) which are most likely to be found in nanofood products divided into three main class: inorganic, surface functionalized materials, and organic engineered nano materials [25].

Inorganic nanomaterials for application in food, food additive, food packaging or storage include ENMs of transition metals, such as silver and iron; alkaline earth metals, such as calcium and magnesium, and non-metals, such as selenium and silicates.Consumer products containing nanosilver, including food, water, and food contact surfaces and packaging materials. Indeed, the use of nanosilver as an antimicrobial, antiodorant, and a (proclaimed) health supplement has already surpassed all other ENMs currently in use in different sectors [26]. The green tea products containing nanoselenium is being marketed, with a number of health benefits resulting from enhanced uptake of seleniume [26]. Nanocalcium salts are subject of patent application for intended use in chewing gums. Nanocalcium and nanomagnesiume salts are also available as health supplements [27].

Nano-iron is available as a health supplement and is used in treatment of contaminated water, where it is claimed to decontaminate water by breaking down organic pollutants and killing microbial pathogens [28].

Researchers reported iron- and iron/zinc-containing nanostructured compound for nutritional application that improved bioavaibility and minimize color changes in finished products [29-30].

Also, nanoemulsions are effective against a variety of food pathogens, including Gram-negative bacteria.

They can be used for surface decontamination of food processing plants and for reduction of surface contamination of chicken skin [31].

Mg plays several vital roles in human biology. Its deficiency has been implicated in regulation of blood pressure. The institute of Medicine of National Academy of Sciences has established Recommended Dietary Allowances which defines the average daily intake that is sufficient to meet the requirements is 420 mg [18, 32].

Mg is ingested as a dietary supplement in the form of MgO and MgOH.

These are insoluble in water, but readily soluble in gastric juice, and the Mg ion becomes bioavailable upon dissolution in the stomach. MgO and MgOH serve as pH control agents in dairy products and in the manufacture of canned vegetables such as peas. They also serve as flow enhancer and anti-caking agents in dry breakfast cereal, salt production, and powder concentrates such as soft-drink mixes [33].

CONCLUSION

Using a combination of nisin, mild heat and MgO NP treatments could successfully control *E. coli* and *S. aureus* in the milk, and the combinations of nisin, heat and MgO NP are synergistic.

Mild heating plus nisin and MgO NP treatment are synergistic, so combined treatments can be developed, which would decrease the temperature and amount of MgO NP required for minimally processed milk during maintaining pathogen control.

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