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Increasing the Shelf Life of Milk by Metal Oxide Nanoparticles and Mild Heat

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ABSTRACT

Background: The spread of pathogenic microorganisms in food and beverage and their resistance to antibiotics have raised major concerns for public health. The aim of this study was to investigate the antimicrobial activity of various metal oxide nanoparticles (NPs) including zinc oxide (ZnO), magnesium oxide (MgO), and iron oxide (Fe_2O_3) NPs against *Staphylococcus aureus (S. aureus)* and *Escherichia coli (E. coli)*. Furthermore, the antimicrobial activity of these NPs in milk was studied along with mild heat. **Methods**: In this experimental study, the antibacterial activity of ZnO, MgO, and Fe_2O_3 NPs were initially evaluated by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods. Later, the antimicrobial effect of these NPs was investigated in milk along with mild heating. To determine the morphological changes in *S. aureus* and *E. coli*, electron microscopy scanning was applied before and after the antimicrobial treatments. **Results:** The MBC and MIC values presented by Fe_2O_3, ZnO, and MgO NPs against pathogenic bacteria showed that MgO NPs were the most potent substances for inhibiting the growth of *S. aureus* and *E. coli*. The results also indicated that use of these NPs had synergistic effects in combination with the heating treatment. Electron microscopy scanning also revealed that treatment with MgO NPs could distort and impair the cell wall of the pathogenic bacteria, leading to the leakage of intracellular components and bacterial death. **Conclusion:** The results suggest that MgO, ZnO, and Fe_2O_3 NPs can be applied for industrial food processing as effective antimicrobial compounds to decrease the temperature required for pasteurizing milk.

Keywords: *Antimicrobial activity; Nanoparticles; Zinc oxide; Magnesium oxide; Iron oxide; Staphylococcus aureus; Escherichia coli*

Introduction

Considering the recent emergence of pathogenic bacteria, which are resistant to a broad spectrum of antibiotics, and the adverse effects of some antibiotics on human health, effective antimicrobial compounds with minimum side effects should be found urgently to eliminate infectious agents from the food and environment (Kimiaee Sadr *et al.*, 2016, Li *et al.*, 2008,

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Magiorakos *et al.*, 2012). Numerous efforts have been made to eradicate multiple- antibiotic-resistant pathogenic bacteria using antimicrobial nanomaterials. The aim was to reduce the food-borne bacteria and life-threatening infections with low toxicity (Li *et al.*, 2008). One of the significant properties of antimicrobial nanoparticles (NPs) is their ability to inhibit the growth of gram-negative bacteria and food-borne pathogen (Akbar and Anal, 2014). Applicability of NPs in different fields of science such as biology, medicine, and pharmacology, especially in medications has attracted much attention. The researchers found that NPs could be employed in food preservation and food packing, leading to increased food safety in industrial food processes (Akbar and Anal, 2014). Metal oxide NPs, a new class of compounds with unique physical and chemical properties, have antibacterial activities against various types of harmful microorganisms. Among NPs, metal oxide NPs, such as ZnO, Fe_2O_3, and MgO are the most considered research subjects (Xie *et al.*, 2011). Application of NPs is not exclusively confined to medicines, rather they are widely used in industrial productions such as cosmetics, plastics, paints, ceramics, and textiles (Rizzo *et al.*, 2013). Metal oxides NPs were reported to have antibacterial activity against microorganism pathogens, such as *Staphylococcus aureus, Escherichia coli*, *Aspergillus niger*, *Saccharomyces spp*, and Mushroom species (Hewitt *et al.*, 2001). However, gram-positive bacteria are more prone to be affected by metal oxide NPs in comparison with gram-negative bacteria (Li *et al.*, 2008). Due to the broad spectrum antibacterial properties of metal oxide NPs, these nanomaterials are considered as potential candidates to be applied in food industry (Stoimenov *et al.*, 2002). The size of NPs was inversely associated with the inhibitory effects of these materials on bacterial growth (Akbar and Anal, 2014). Prophylactic strategies, rapid diagnosis, and advanced antibiotic therapy have significantly improved the life of human beings in recent years. However, antibiotic resistance is still the main enigma in public health that urges scientists to seek more effective compounds against multi-drug resistant bacteria with low toxicity in order to overcome the bacterial resistance (Rizzo *et al.*, 2013).

The spread of infectious diseases caused by microorganisms in the food and increased bacterial resistance to antibiotics have arisen a major problem for the public health (Makhluf *et al.*, 2005). Hence, new compounds should be explored and designed to fight against such bacteria. Metal oxide NPs have evolved as new antimicrobial agents, which can be potentially used against food-borne pathogens (Akbar and Anal, 2014, Dastjerdi and Montazer, 2010). Considering the heat resistance, antibacterial activity, targeting abilities, high durability, and thermal conductivity of metal oxide NPs, they are very promising in the discovery and delivery of bioactive molecules for food-borne bacteria, spores, and viruses. Therefore, the present study aimed to monitor the antibacterial activity of some metal oxide NPs including ZnO, Fe_2O_3, and MgO against food-borne bacteria in the culture medium and milk.

Materials and Methods

Bacterial strains, medium culture, and materials: The strains of bacteria used in this study were *Escherichia coli (E. coli)* PTCC1394 and *Staphylococcus aureus (S. aureus)* PTCC1431, which were both obtained from the culture collection of Iran. Stock cultures were maintained at −70 °C until use. Bacteria were active on tryptic agar at 37 °C and maintained at 0–2 °C before the experiments. Furthermore, MgO NPs (with the particle size of 20-30 nm) were prepared from US Research Nanomaterials Inc (Houston, TX); ZnO NPs were prepared from Teconan (Navarra, Spain) with the particle size of 20–25 nm; and Fe_2O_3 NPs (>50 nm particle size) were obtained from Sigma Chemical Company (Darmstadt, Germany). The soluble form of all NPs was prepared in double-distilled water and then sonicated in an ultrasonic bath (S30H Elmasonic, Germany) for 20 min. As a result, the homogenous colloidal suspension was achieved for each nanoparticle (Mirhosseini and Firouzabadi, 2013).

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Antimicrobial effect of NPs: The antibacterial activity of all NPs, namely, ZnO, Fe_2O_3, and MgO was evaluated against *E. coli* and *S. aureus*. For this purpose, the spot-on-lawn assay was employed to measure the potency of NP types in the suppression of bacterial growth. Briefly, 20 µL of the colloidal suspensions of NPs at concentrations of 0, 0.65, 1.25, 2.5, and 5 mg/ml were spotted onto tryptone soya agar (TSA)soft-agar lawns (0.6%). Later, they were inoculated by 10^7 bacteria/mL *E. coli* or *S. aureus* according to 0.5 McFarland standards. Each plate of bacteria was incubated at 37 °C for 24 h. Afterwards, the inhibition zone around each spot was calculated to measure the inhibitory effect of each metal oxide NP (Mirhosseini and Firouzabadi, 2013).

Minimum inhibitory concentration (MIC) determination of NPs: The minimal concentration of a given antimicrobial agent that inhibits the growth of microorganisms is known as the MIC (Stoimenov *et al.*, 2002). To measure the MIC of ZnO, Fe_2O_3, and MgO NPs colloidal suspension from each NP was prepared in which the concentration of each NP was 100 mg/ml. At first, 1 ml of TSB media was added to the tubes and then 1 ml of each NP was poured into the tube. In the next step, a serial two-fold dilution method (100- 50- 25-12.5-6.25,….mg/ml) was applied to achieve the lowest concentration of each NP to inhibit the growth of bacteria, starting from tube 1 to tube 7. The 8^{th} tube was considered as the control tube. Later, 100 µL of the 10^7 bacteria/mL corresponding to 0.5 McFarland standards of either *S. aureus* or *E. coli* was added to all tubes. Subsequently, all tubes were incubated at 37 °C overnight. The rate of bacterial growth was determined by the measurement of optical density (OD) at 620 nm performed by the spectrophotometry (Optizen 2120 UV Plus, Korea) analysis. The obtained value for each NP was designated as the MIC (Stoimenov *et al.*, 2002, Xie *et al.*, 2011).

Minimum bactericidal concentration (MBC) determination of NPs: The MBC is characterized by determination of the lowest concentration of an antibacterial agent that can reduce viability of the initial bacterial inoculum by ≥ 99.9%. The value of MBC for each test tube, without visible growth, was obtained by subculturing 50 µl on tryptone soya agar (TSA). After incubation at 37 °C for 24 h, the MBC was defined as the lowest concentration leading to a negative subculture or giving the presence of only one colony after the incubation process (Stoimenov *et al.*, 2002).

Determination of the effect of NPs and heat on bacterial load of milk: Traditional cow's milk was used for the present study. Metal oxide NPs namely, ZnO, Fe_2O_3, and MgO were dissolved in milk to reach the concentration of 1.5 mg/ml. Milk samples were then inoculated with 100 µl of the mixed bacteria for each strain (equivalent to 10^7 CFU mL^{-1}). The heating process was carried out in a water bath at 100 °C in which the temperature was controlled by the insertion of a thermometer in milk containing NPs. During the thermal treatment, milk samples were homogeneously agitated to obtain uniform distribution of the inoculums. Once the temperature of milk samples reached the target treatment temperature of 50-60 °C, samples were removed from water bath and immediately transferred into the ice box (Premanathan *et al.*, 2011).

One milliliter of the treated samples was dissolved in 9 ml of 0.2% (w/v) peptone water and then serially diluted (10^{-1}–10^{-5}) in 0.1% peptone water. Mannitol salt agar (MSA, Merck, Darmstadt, Germany) and eosin methylene blue (EMB, Merck, Darmstadt, Germany) agar were employed for the separating and counting *S. aureus* and *E. coli*, respectively. Microscopic analysis of bacteria was accomplished by the Gram stain smear. The biochemical and sugar fermentation tests in phenol read broth were also performed for the identification and confirmation of the bacterial isolates contained in milk samples (Brayner *et al.*, 2006, Phillips, 1993).

Analysis of cell morphology by SEM: Cultivated bacteria namely, *S. aureus* and *E. coli*, at the mid-log phase of growth corresponding to 0.5 McFarland standards, were treated with 1.5 mg mL^{-1} MgO NPs for 18 h. One milliliter of the

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treated and untreated bacterial suspensions were centrifuged at 10,000 rpm for 5 min and the bacterial sediments were dissolved in 100 µL tryptic soy broth. Afterwards, 20 µL of the bacterial suspensions were put on glass coverslips. The glass coverslips were air-dried for 45 min and then fixed with a solution containing 2.5% glutaraldehyde and 0.1 M imidazole buffer solution (pH 7.2) for 2 h. Later, the fixative solution was replaced by 0.1 M imidazole buffer, followed by dehydration with an ascending ethanol series (50, 80 and 100%). The coverslips were placed on SEM stubs by means of carbon adhesive disks, which were then sputter-coated with a thin layer of gold using Sputter Coater device (DSR1, Nano-Structured Coatings Company, Iran). Finally, digital images of the treated and untreated *S. aureus* and *E. coli* were captured using the SEM (Phenom ProX, Netherland) that was operated at an accelerating voltage of 10 kV and instrumental magnifications of 35,000 (Jin and He, 2011).

Data analysis: The values obtained in experiments were presented as mean ± standard deviation (SD). One-way analysis of variance (one-way ANOVA) was applied followed by Duncan's multiple range test (Duncan 1955) in order to compare the significance of values between different groups. The analysis of the data was conducted by the SPSS software (version 16, SPSS, Chicago, USA). The level of significance was set at P-value < 0.05.

Results

Antimicrobial effect of NPs: The results showed that the suspension solutions of ZnO, Fe_2O_3, and MgO NPs did not affect the growth rate of *E. coli* and *S. aureus* in a solid medium.

MIC determination of NPs: The results indicated that the MIC value for MgO NPs against *E. coli* was 2.5 mg/ml, which showed a potent inhibitory effect on bacterial growth. The MIC value for ZnO NPs against *E. coli* was 4.5 mg/ml, indicating a moderate inhibitory effect on bacterial growth. The MIC value for Fe_2O_3 NPs against *E. coli* was 80 mg/ml, exhibiting a weak inhibitory effect on the growth of the bacteria. Our findings revealed that ZnO and MgO NPs had acceptable impacts on inhibiting the growth of *S. aureus.* The MIC values for MgO and ZnO NPs against *S. aureus* were 2 mg/ml and 2.2 mg/ml, respectively. It can be concluded that both MgO and ZnO NPs have the same potency against the bacterial growth when applied for the infection caused by *S. aureus*. The MIC value for Fe_2O_3 NPs against *S. aureus* was 45 mg/ml, showing a weak potency to inhibit the growth of *S. aureus.*

MBC determination of NPs: The result showed that the MBC values of MgO, ZnO, and Fe_2O_3 NPs against *E. coli* were 3 mg/ml, 25 mg/ml, and 90 mg/ml, respectively. Furthermore, the MBC values for MgO, ZnO, and Fe_2O_3 NPs against *S. aureus* were 2.5 mg/ml, 5 mg/ml, and 50 mg/ml, respectively. According to the results of the MBC, it seems that *S. aureus* is more susceptible to be influenced by NPs.

The effect of NPs and heating process on milk: Figure 1 illustrates the survival of *E. coli* exposed to MgO, ZnO, and Fe_2O_3 NPs, as well as the heat or combination of NPs and heat. The treatment of milk with MgO NPs significantly decreased the growth rate of *E. coli* ($P < 0.05$); however, application of ZnO and Fe_2O_3 NPs did not considerably reduce the growth of *E. coli* as compared with the control sample for 24 h ($P > 0.05$). Additionally, co-treatment of milk with the heat and MgO, ZnO, and Fe_2O_3 NPs had synergistic effects on inhibition of the bacterial growth when compared with the individual treatment ($P < 0.05$).

Figure 2 depicts the survival of *S. aureus* treated with MgO, ZnO, and Fe_2O_3 NPs, as well as the heat or in the combination of heat and NPs. Similar to *E. coli*, the efficacy of the anti-staphylococci activity of NPs was dependent on the type of NPs and temperature. Our findings showed that combinatory treatment of milk with heat and NPs enhanced the antibacterial activity of MgO, ZnO, and Fe_2O_3 NPs against *S. aureus* significantly ($P < 0.05$). Furthermore, *S. aureus* was more prone to be affected by treatment with MgO, ZnO, and Fe_2O_3 NPs when compared with *E. coli*. This effect

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would be more tangible when the same amount of NPs or temperatures (during 8-24 h) was exposed to both types of bacteria, leading to a further decrease in the growth rate of *S. aureus*.

Analysis of cell morphology by scanning electron microscopy (SEM): Considering the results obtained from the SEM analyses, the morphological features of *S. aureus* exposed to MgO NPs were significantly distorted since some irregularities and heterogeneities were evident in this bacterium (**Figure 3b**). Conversely, *S. aureus* exposed to the control colloidal solution exhibited a regular shape as well as a smooth surface with spherical grape-like clusters (**Figure 3a**). Most of *S. aureus* treated with MgO NPs were completely ruptured as depicted by the atypical and disintegrated cell wall. However, some bacteria treated with MgO NPs only showed some abnormalities in bacterial shape.

Figure 4 displays the SEM analyses of *E. coli* that received no treatment (**Figure 4a**) and bacteria treated with MgO NPs (**Figure 3b**) for 18 h in TSB. Regarding the control bacteria, the untreated bacteria showed rod-like forms in regular sizes with an intact cell wall (**Figure 4a**). Treatment of bacteria with MgO NPs impaired *E. coli* and caused some deformities in cell surface and homogeneity of the cell wall significantly (**Figure 4b**).

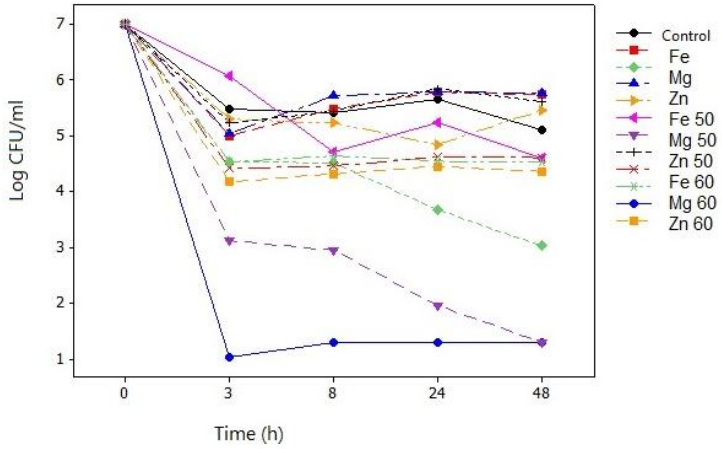


Figure 1. Effects of MgO, ZnO and Fe_2O_3 NPs and heat on the viability of *E. coli* at high inoculum levels in milk.

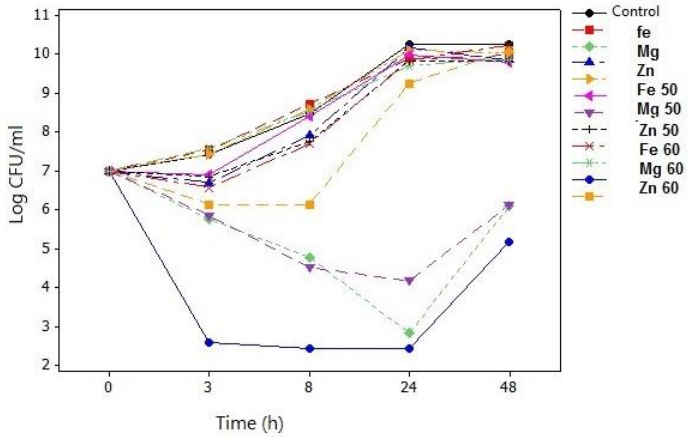


Figure 2. Effects of MgO, ZnO and Fe2O3 NPs and heat on the viability of S. aureus at high inoculum levels in milk.

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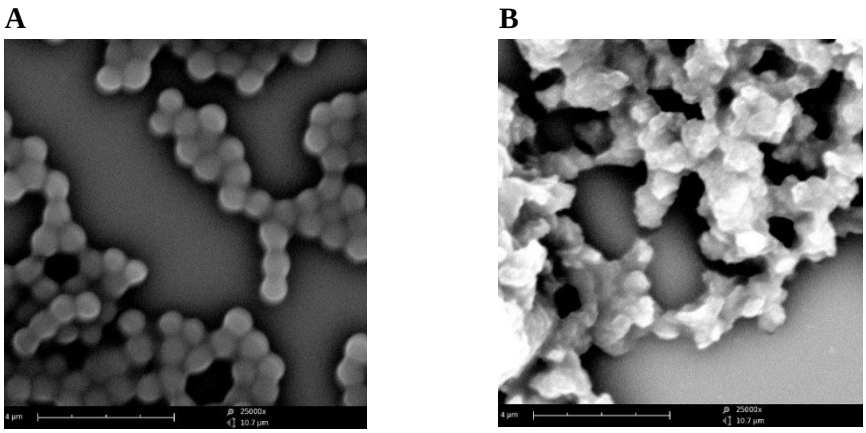


Figure 3. Scanning electron micrographs of S. aureus following treatments with MgO NP. Bacteria were incubated with a: TSB alone (control), b: 1.5 mg mL-1 MgO NP

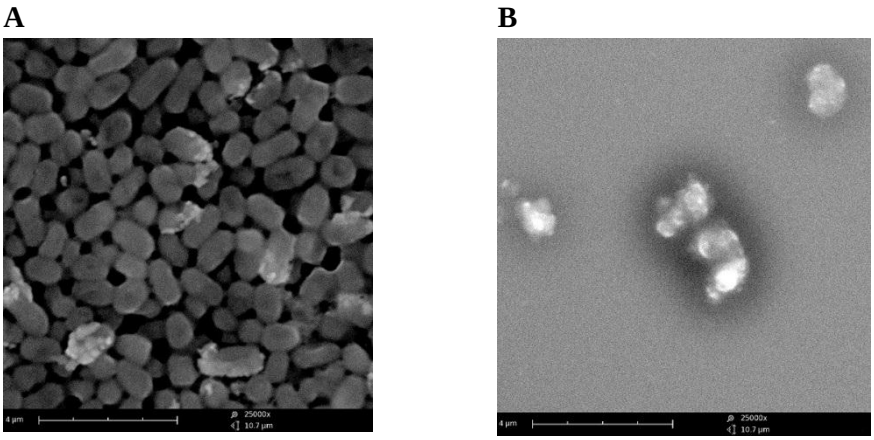


Figure 4. Scanning electron micrographs of E. coli following treatments with MgO NP. Bacteria were incubated with a: TSB alone (control), b: 1.5 mg mL-1 MgO NP

Discussion

In the present study, antimicrobial effects of MgO, ZnO, and Fe_2O_3 NPs were investigated in liquid and solid media. The results indicated that antibacterial activity of the NPs was dependent on the type of NPs as well as the concentration of NPs used in this research. In this study, MgO, ZnO, and Fe_2O_3 NPs at a concentration of 1.5 mg/ml were selected for further analysis of bacterial load in milk.

Our findings showed the reduced growth of all bacterial strains inoculated into milk when exposed with MgO, ZnO, and Fe_2O_3 NPs at a concentration of 1.5 mg/ml. We indicated that, compared with the control group, MgO NP as had more inhibitory effects on all bacterial strains in milk during 48 hours ($P < 0.05$). Furthermore, the heating process in the presence of MgO NPs diminished bacterial growth synergistically compared with the individual treatment by either temperature therapy or NPs' treatment ($P < 0.05$). On the other hand, ZnO and Fe_2O_3 NPs had the same effects in comparison with the control, showing no significant impact on the bacterial growth ($P >$

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0.05). Increase of temperature in the presence of either ZnO or Fe_2O_3 NPs elevated the inhibitory effects of these two NPs on bacterial growth significantly ($P < 0.05$). Consistent with our findings, Stoimenov et al. showed that MgO NPs had powerful antibacterial activity against *Escherichia coli* and *Salmonella Stanley*, which are considered as food-borne pathogens. Based on the literature, MgO NPs in the form of powder or liquid are effective in eliminating gram-positive bacteria, gram-negative bacteria, spores, and viruses. MgO NPs have been widely used as antimicrobial agents in eradication of the microorganisms (Stoimenov *et al.*, 2002). In another study, Makhuf et al. investigated the mechanisms underlying the antibacterial activity of MgO NPs. They concluded that the generation of reactive oxygen species, as a result of treatment with MgO NPs, as well as the interaction of these NPs with the cell wall of the bacteria, made these nanomaterial bona fide for destructing the bacterial cell membrane. This is because they are able to cross the bacteria cell wall and impair its metabolism (Makhluf *et al.*, 2005). Dastjerdi and Montazer also examined the antimicrobial effect of ZnO NPs on the growth rate of bacteria. They revealed that ZnO NPs had the antibacterial effect against food-borne *E. coli* and enterotoxigenic *E. coli* (ETEC). The studies conducted in this regard proposed that ZnO NPs may be useful for agricultural products due to their low cost and UV resistance (Dastjerdi and Montazer, 2010). In a similar study, Wilczynski et al. examined the antibacterial activity of ZnO NPs against *E. coli* and *S. aureus.* They concluded that these NPs could inhibit the bacterial growth when treated at a concentration of 10 mM (Wilczynski, 2001). Compared with other studies conducted in this area, our data showed that magnesium and zinc NPs had the highest antimicrobial activity in inhibiting the growth of *S. aureus* and *E. coli* strains.

Mirhosseini and Barzegari Firouzabadi investigated the antibacterial activity of ZnO NPs against *Listeria monocytogenes* and *Bacillus cereus*. They showed that ZnO NPs had inhibitory effects on *L. monocytogenes* and *B. cereus* in milk (Mirhosseini and Barzegari Firouzabadi, 2015). It is thought that ZnO NPs are able to destroy proteins and lipids of the bacterial cell membrane, leading to the rupture of cell membrane, discharge of intracellular contents, and ultimately the death of bacterial cells. Accordingly, production of hydrogen peroxide and Zn^{2+} ions was proposed to be involved in antimicrobial mechanisms of ZnO NPs in killing the bacteria (Mirhosseini and Firouzabadi, 2013, Xie *et al.*, 2011). The results indicated that MgO NPs had the maximum inhibitory effect against *E. coli* and *S. aureus*. The SEM analysis revealed that treatment of milk with MgO NP caused significant changes in cell morphology and membrane integrity (Figure 3, 4). The viability test of *S. aureus* or *E. coli* treated with MgO NPs showed that they were no longer cultivable, implying the destruction of bacterial membrane or disintegration of the bacterial cell wall. To date, the molecular mechanisms, through which MgO NPs are capable of killing bacteria, are still unclear. However, a number of studies suggested that production of reactive oxygen species (ROS), intercalation of NPs into the cell wall of bacteria, and alkaline reactions were potential mechanisms by which MgO NPs could exert their bactericidal effects (Jin and He, 2011, Tang and Lv, 2014).

In this research, we examined the impact of MgO, ZnO, and Fe_2O_3 NPs on the viability of *E. coli* and *S. aureus* bacteria in milk under different temperatures. Our findings indicated that the temperature of 60 °C had the highest efficacy in eradicating the above-mentioned bacteria. We also found that the effect of co-treatment with NPs and heat was more pronounced in comparison with individual treatment. Firouzabadi et al. also showed the antibacterial effect of ZnO suspensions containing 0.3% citric acid against the pathogenic bacteria such as *L. monocytogenes*, *E. coli*, *S. aureus*, and *B. cereus* in mango juice.

Their findings exhibited that ZnO NPs had an inhibitory effect on the growth of all bacterial strains when used in mango juice as a culture medium for 24 h (Firouzabadi *et al.*, 2014). A study carried out by Mirhosseini and Arjmand

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showed the antibacterial activity of ZnO suspensions containing 1% acetic acid against the pathogenic bacteria including *L. monocytogenes*, *E. coli*, *S. aureus*, and *B. cereus* in sheep meat. Moreover, ZnO NPs decreased the initial growth of all inoculated bacteria in meat significantly (Mirhosseini and Arjmand, 2014). Nanomagnetic NPs can be used as a health supplement and applied for the treatment of polluted water, since they can detoxify water by degradation of the organic contamination and eradication of microbial pathogens. Studies highlighted that iron- and iron/zinc- containing nanomaterials could enhance the bioavailability of nutrition and also minimize color change in biologically cultivated products (Sekhon, 2010). ZnO is one of the five types of inorganic compounds considered non-toxic for human beings according to the U.S. Food and Drug Administration (FDA). Zinc salts are widely employed as supplementary nutrition for treating zinc inadequacy. The maximal nutritional allowance approved for adults is allegedly 40 mg per day (Mirhosseini and Barzegari Firouzabadi, 2015).

Magnesium plays a crucial role in the biological process of the human body as its deficiency is involved in uncontrolled blood pressure.

Regarding the guidelines presented by the National Academy of Sciences, the recommended daily allowance (RDA) for magnesium is 420 mg for adults considering their age, weight, and gender (Jin and He, 2011). Magnesium is absorbed as a nutritional supplement in the form of MgO and MgOH. Although these compounds are insoluble in the water, they are readily absorbed in the gastrointestinal tract when dissolved in gastric juice and bioavailability of magnesium ion would be increased upon being dissolved in the stomach. Magnesium oxide and magnesium hydroxide can act as pH regulators in dairy products and canned vegetables such as peas. They are also used as the emulsifier and anti-caking agents in dry breakfast cereal, salt products, and powder beverages such as soft-drink mixes (Krishnamoorthy *et al.*, 2012).

Conclusions

Ferro oxide, magnesium oxid, and zinc oxid NPs exhibit antibacterial activity against both *S. aureus* and *E. coli* bacteria; however, their bactericidal effects were more highlighted on *S. aureus*. Among NPs used in our study, MgO was the most powerful NP concerning the antibacterial effect of other nanomaterials namely, ZnO and Fe_2O_3. It should be noted that the combinatory treatment of milk with the heating process and use of NPs increased bactericidal effects on *E. coli* and *S. aureus* considerably. This can provide an opportunity for the food industry to use mild heating instead of high-temperature treatment and can preserve milk's nutritional and organoleptic properties.

Authors' contribution

Mirhosseini M and Dehestani R designed the research. Mirhosseini M conducted research; Dehestani R analyzed data. Both authors wrote the paper. Mirhosseini M had primary responsibility for the final content. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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