Journal of Biology and Today's World
ISSN 2322-3308
http://www.journalbio.com

Received: 16 June 2014 • Accepted: 10 September 2014

Research

doi:10.15412/J.JBTW.01031005

Combined effect of mild heat and zinc oxide nanoparticle treatment for inactivating *Escherichia coli*, *Listeria Monocytogenes* and *Staphylococcus aureus* in milk

Mahboubeh Mirhosseini[1,2*], Narjes Mohammadi[1], Maryam Shirzad[1]

[1] Department of Biology, Payame Noor University, Iran

[2] Nano Structured Coatings Institute, Yazd Payame Noor University, PO Code 89431-74559 Yazd, Iran

*correspondence should be addressed to Mahboubeh Mirhosseini, Mahboubeh Mirhosseini, Department of Biology, Payame Noor University, Yazd, Iran; Tell: +983515216134; Fax: +983515212627; Email: m.mirhosseini@pnu.ac.ir.

ABSTRACT

Recently, the use of metal oxide nanoparticles to control and prevent the spread of microorganisms is an expanding field of research. The objective of this study was conducted to validate combined heat and ZnO nanoparticle (NP) treatments for inactivating *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* in milk. A mixture of three strains of *E. coli*, *L. monocytogenes* and *S. aureus* was inoculated onto the milk samples. A combinations of various concentrations of ZnO NP [0%, 0.025%, 0.05% (W/V)] and various temperatures (50 °C and 60 °C) was investigated. Following the treatment, milk samples were stored at room temperature and enumerated on 0.5, 1, 2 and 3 days. Heat and ZnO NP treatments were synergistic. Inhibitory effects of these combined treatments on the tested foodborne pathogens were also effective during storage.

Key words: ZnO nanoparticles, combined effect, Escherichia coli, Listeria monocytogenes, Staphylococcus aureus

1. INTRODUCTION

Outbreak of food-borne pathogens such as *E. coli*, *Salmonella* spp., and *L. monocytogenes* in food safety has attracted the public attention. Thus, it is necessary to develop new antimicrobial agents to ensure food safety and extend shelf life. In recent years, use of inorganic antimicrobial agents to control microbes has attracted great interest in non-food applications (1, 2). An advantage of applying formulaic organic materials to control prevents the spread of microorganism toxicity and susceptibility to high temperature and pressure (3-6). Therefore, further research is necessary to develop new antimicrobial materials such as: metal oxides (4, 6, 7). These new antimicrobial materials are effective in controlling the growth of various microorganisms (6, 8). They are non- toxic and stable under harsh processing conditions (5-9). Metal or metal ions are also essential for human body and animal cell, which are needed for more than 300 biochemical reactions in the body (2, 10, 11). Among inorganic material, metal oxides nanoparticles are of the important links of nanotechnology to the science (6). Reduction of material to nano size leads to the obtaining of compounds with improved physical, chemical, and biological properties that can be used in the medical and industrial fields (6, 12). Nanoparticles have attracted great attention owing to their distinct physical, chemical, and biological properties that are unavailable in conventional macroscopic materials. Zinc oxid (ZnO) belonging to a group of metal oxides is characterized by photo catalytic and photo-oxidizing abilities against chemical and biological species. Availability of a wide range of nanostructures makes ZnO an ideal material for nanoscale optoelectronic and piezoelectric nano-generators as well as biotechnology (13). Food color and texture are negatively affected by thermal processing (14). Also, the recommended maximum dietary allowance for zinc is 40 mg per day in adults (15), which is equivalent to 100 mL of daily intake of milk if 0.4 mg of ZnO is used per mL of food (16). This issue is an important disadvantage of these nanoparticles for use as an antibacterial agent in the food industry. Therefore, further research is necessary to investigate the efficacy of ZnO at a lower concentration

level on inactivating pathogens in foods. Thus, it is important to evaluate possible hurdle technologies or combination treatments that exploit synergistic interactions between different preservation treatments in order to control the pathogen growth. The hurdle technology concept promotes a combination of low-intensity preservation treatments that have additive or synergistic antimicrobial effects, as a means of reducing impact on the sensory and nutritive properties of food (17). However, to date, there has been no study on the combined effect of heat and ZnO NP treatment on the safety of milk. In this study, the combined effect of heat and ZnO NP on killing *L. monocytogenes*, *E. coli*, and *S. aureus* was examined in milk.

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

The following bacterial strains were used in this study: *L. monocytogenes* [PTCC1163], *E. coli* [PTCC1394], and *S. aureus* [PTCC1431]. The mentioned bacteria were obtained from the culture collection of the I.R. Department. Zinc oxide nanoparticles (particle diameters: 20–25 nm, purity: 99.98%) were purchased from Teconan (Spain) (16). Each strain was cultured in a 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 suspended again in buffered peptone water, corresponding to approximately 10^7–10^8 CFU ml^{-1} and mixed cocktails were prepared by blending the equal volumes of each test strain.

2.2. Sample treatment

The milk samples containing (0%, 0.025%, and 0.05%) ZnO nanoparticles were prepared. 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 the heat treatment, the 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 and 60 °C the glass bottles were removed and immediately cooled in crushed ice. The samples were stored at room temperature and examined on 0.5, 1, 2 and 3 days, respectively, to allow for the recovery and then enumeration of injured cells. All the experiments were repeated three times (14).

2.3. Bacterial enumeration

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. Mannitol salt agar (MSA, Merck, Darmstadt, Germany) was used for the isolation and enumeration of *S. aureus*. Eosin methylene blue (EMB, Merck, Darmstadt, Germany) agar was employed for the isolation and identification of *E. coli* and *Listeria* selective agar was applied for isolating and enumerating of *L. monocytogenes*. Microscopic examination of the isolates was performed by staining smears according to the Gram method. Identification and characterization of bacterial isolates up to the species level were implemented using various biochemical and sugar fermentation tests. Carbohydrate fermentation tests detect the ability of microorganisms to ferment a specific carbohydrate. Fermentation patterns can be used to differentiate among bacterial groups or species. Fermentation reactions are detected by the color change of a pH indicator when acid products are formed. This is accomplished by adding a single carbohydrate to a basal medium containing a pH indicator. The Carbohydrate Fermentation test uses Phenol Red Broth to test for the fermentation of different sugars (9, 18).

2.4. pH measurement

The pH values of the samples were determined using a pH meter (827 pH lab, UK) after adding ZnO, following heat treatment and during the time course of the microbial incubation period.

2.5. Statistical analysis

All the experiments were repeated three times. Analysis of Variance was carried out ($P < 0.05$) (SPSS) for a completely randomized design (ZnO concentration, temperature, storage time), with means separated by a least squares means test (PDIEF, a pair wise t-test).

3. RESULTS AND DISCUSSION

3.1. pH changes in milk samples

Milk samples without ZnO NP had pH of about 6.8, and there was no significant decrease in pH values with the increasing concentration of ZnO NP ($P > 0.05$). There was no significant difference among pH values following the heat treatments at any treatment temperature ($P > 0.05$). During the 3 day storage time, pH significantly changed at any concentration of ZnO NP ($P > 0.05$). However, pH significantly dropped in the samples without added ZnO after 1 day of storage at room temperature ($P < 0.05$) as a result of microbial growth (Figure 1).

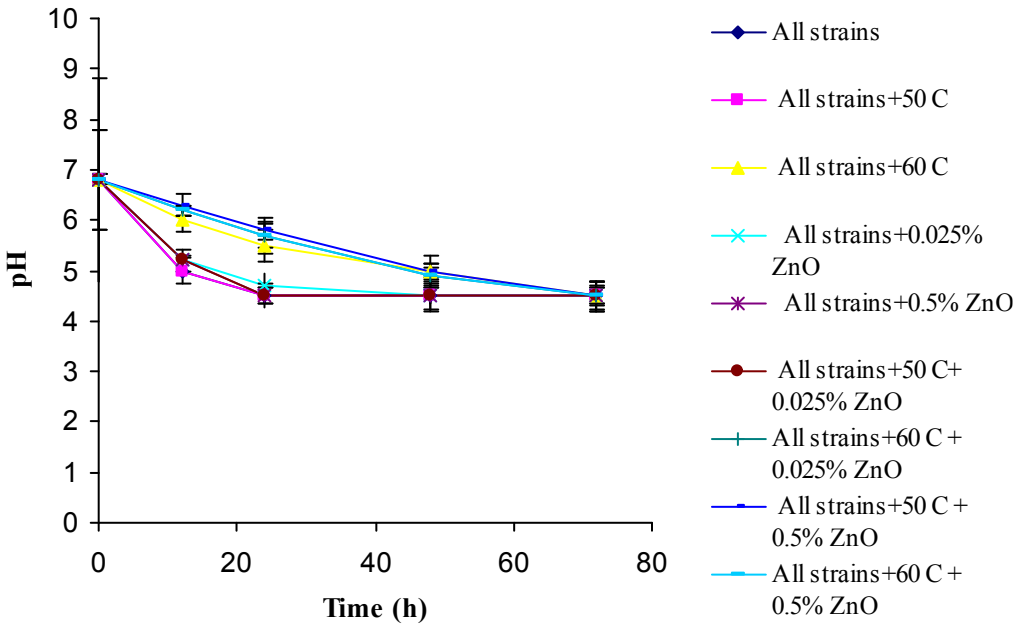

Figure 1. pH Changes in inoculated milk samples on 3 days of storage at room temperature.

3.2. Combined effect of ZnO NP and heat on inactivating E. coli

Figure 2 shows the survival curves of *E. coli* in inoculated milk during storage. The initial level of *E. coli* was approximately 10^7 CFU ml^{-1}. Surviving *E. coli* grew in the untreated samples during room temperature storage and reached high levels (‡9.17 log) after 0.5 day of storage. When ZnO NP was added without heat treatment, there was less than 1-log reduction for 0.05% ZnO NP concentration after 0.5 day of storage (Figure 2). When only heat was applied, 2-log reduction was resulted, as treatment temperatures increased to 60 °C (Figure 2). When 0.025% ZnO was combined with 50 or 60 °C heating treatment, 2.37- and 5.58-log reductions were observed (Fig. 2). The combination of 0.05% ZnO and heating to 50 and 60 °C resulted in 4.72 and 6.06-log reductions, respectively after 0.5 day of storage (Figure 2). Surviving *E. coli* grew in the untreated samples during room temperature storage and reached high levels (‡11.3 log) after 1 day of storage. In the samples with added ZnO NP, *E. coli* was significantly reduced ($P < 0.05$). When 0.025% ZnO was combined with 50 or 60 °C heating treatment, 1.57- and 2.65-log reductions were observed (Figure 2). The combination of 0.05% ZnO and heating to 50 and 60 °C resulted in 2.42 and 2.9-log reductions, respectively (Figure 2).

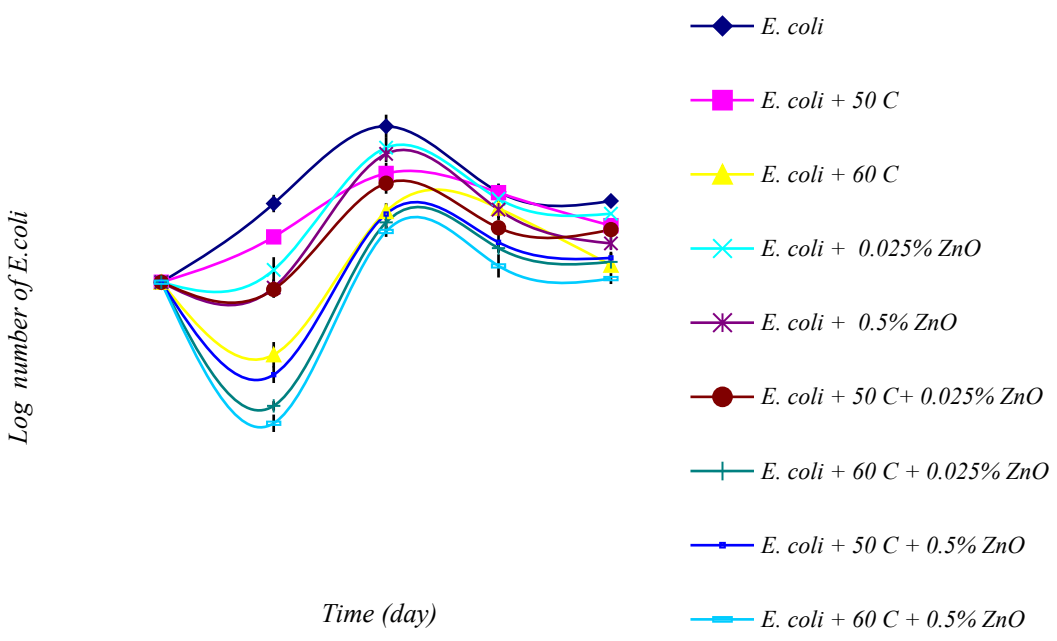

Figure 2. Effect of ZnO NP and heat treatment against *E. coli* [PTCC1394] inoculated in milk

Inhibitory effects of ZnO NP and heat were maintained during storage at room temperature. The samples containing ZnO NP (0.025 and 0.05%) heated at either 50 or 60 °C had increased levels of *E. coli* after 3 days of storage (Fig. 2). Heating temperature appeared to be a more critical factor for inactivating *E. coli* and maintaining reduced levels than concentration of ZnO NP.

3.3. Combined effect of ZnO NP and heat on inactivating L. monocytogenes

Fate of *L. monocytogenes* inoculated into the milk is shown in Fig. 3. Like *E. coli*, *L. monocytogenes* began to grow after heat treatment in the samples containing no added ZnO NP (Fig. 2). Without added ZnO NP, *L. monocytogenes* counts were reduced from 7 to 5.43 and 0.12 log cycles after heating to 50–60 °C, respectively. Surviving *L. monocytogenes* grew in the heated milk samples (50–60 °C) and reached 6.65 and 1.95 log after 1 day of storage at room temperature, respectively. Although heating at 60 °C reduced the initial load of *L. monocytogenes* 7 log, surviving bacteria was multiplied by 2.74 log within 3 days.

When ZnO NP was added to the samples, effectiveness of heating treatments in reducing *L. monocytogenes* levels was significantly enhanced ($P < 0.05$). At the levels as low as 0.025% ZnO NP was combined with heating and *L. monocytogenes* levels remained at reduced values during storage (Figure 3). Combined treatment with ZnO NP (0.025%) and heat (50 and 60 °C) resulted in 2.38 - and 5.47-log reductions (Figure 3). Adding 0.05%, ZnO NP with heating to 50 and 60 °C led to 5.5 and 6.05-log reductions, respectively (Figure 3). 0.05% ZnO NP was more effective and when combined with heating at 50–60 °C (Figure 3). This particular set of treatments may be more effective for controlling *L. monocytogenes* than *E. coli* in this type of product (Figure 3).

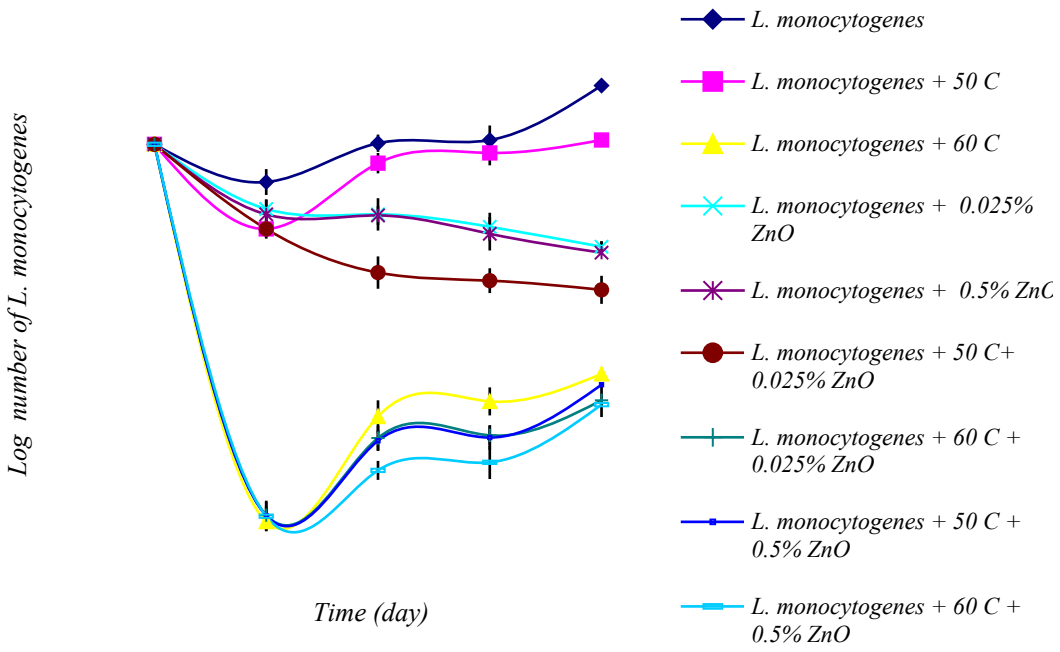

Figure 3. Effect of ZnO NP and heat treatment against *L. monocytogenes* [PTCC1163] inoculated in milk.

Levels of *L. monocytogenes* during storage remained low in the samples treated with both ZnO NP and heat. The *L. monocytogenes* levels in the 0.05% ZnO NP and heat samples dropped to levels of 2.54 and 2.17 log after 3 days of storage at room temperature.

3.4. Combined effect of ZnO NP and heat on inactivating S. aureus

Effect of combined heat and ZnO treatments on the survival of *S. aureus* is shown in Fig.4. Levels of *S. aureus* dropped by 5 and 3.56 log cycle after heating to 50 and 60 °C in the samples to which no ZnO NP had been added (Fig. 4). The microbe grew somewhat in the heat-treated samples after 1 day of storage at room temperature. Levels of *S. aureus* significantly decreased ($P < 0.05$) with the combined treatments of heat and ZnO NP. Adding ZnO NP (0.025%) reduced *S. aureus* to 3 and 6.87 log cycles at 50 and 60 °C heating, respectively (Figure 4), indicating that added ZnO NP at this level was more effective for controlling *S. aureus* than either *E. coli*. Combined treatments of ZnO NP (0.05%) and heat resulted in 6.88 and 6.9-log reductions at 50 and 60 °C of *S. aureus*, respectively (Figure 4). These results indicated that *S. aureus* was more sensitive to the combined treatments of ZnO NP and heat compared with *E. coli*. The combined treatments of heat and ZnO NP was effective for controlling the growth of *S. aureus* in the milk samples during the 3 day storages at room temperature, with

microbial levels remaining low throughout the storage period (Figure 4). With heat treatment at 50 and 60 °C, counts of *S. aureus* were reduced to 4.021 and 1.36 log during room temperature after 3 days. (Figure 4). When 0.025% and 0.05% ZnO were added, counts of *S. aureus* continued to drop during room temperature after 3 days of storage (Figure 4).

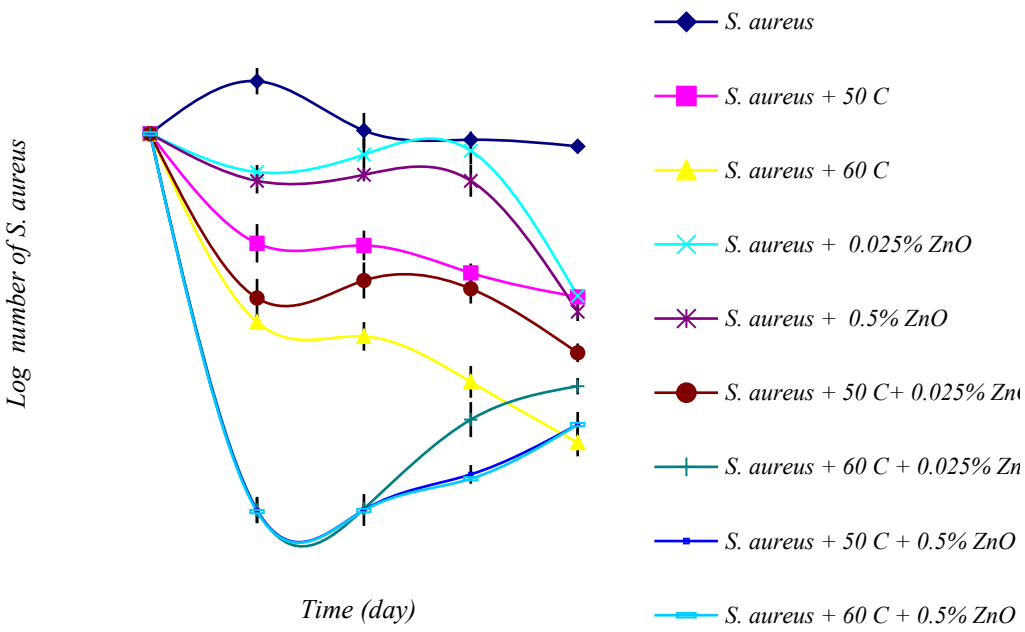

Figure 4. Effect of ZnO NP and heat treatment against *S. aureus* [PTCC1431] inoculated in milk

To apply hurdle technology to food processing, sensory quality must be also considered when determining the appropriate microbial intervention strategies (17). However, some hurdles influence sensory qualities of products, such as color, flavor and texture. In this study, heat and ZnO NP treatments were synergistic and effective in reducing the levels of *E. coli*, *L. monocytogenes* 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 ZnO NP treatment. 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.

In this study, *S. aureus* was more sensitive to the combined treatments of mild heat and ZnO NP compared with *E. coli* or *L. monocytogenes* (Figs. 2, 3, 4). Combined effect of mild heat with relatively low levels of ZnO NP showed synergistic effects in reducing food-borne pathogens in this study, indicating that the antimicrobial effect of ZnO might be enhanced with mild heat treatment. ZnO NPs are believed to destruct lipids and proteins of the bacterial cell membrane, resulting in the leakage of intracellular contents and ultimately death of bacterial cells (19, 20). In addition, generation of hydrogen peroxide and Zn^{2+} ions are suggested to be key antibacterial mechanisms of ZnO NPs (9).

Shin et al., demonstrated that using a combination of mild heat and acetic acid treatment can successfully control *E. coli* O157:H7, *L. monocytogenes* and *S. typhimurium* in pickled asparagus, and effective treatments can be selected to reduce adverse effects on color which occurs during product storage (14). Similar antibacterial effects on reducing pathogen survival in other acidified food samples have been observed by combining mild heating with acid addition (21). Anellis et al. reported that low pH increased the effectiveness of heating on inactivating *Salmonella* spp. in liquid whole eggs (22). To obtain a log reduction of foodborne pathogens in milk, higher storage temperature is desirable, which may be explained by an alteration in the permeability of microbial membranes as a result of increasing temperature (14). Currently, there are very few reports, related to the application of NPs in food safety. For example, Zinc oxide (ZnO) quantum dots were used as the antimicrobial agents in liquid egg white samples. ZnO quantum dots could significantly inhibit or reduce *L. monocytogenes* and *S. enteritidis* in liquid egg white (14, 15). Similar inhibitory effects of ZnO NPs on reducing *S. aureus* and *E. coli* in milk samples were observed in (15, 16). Firouzabadi et al reported that ZnO NP suspension containing citric acid can provide an approximately 5-log reduction in *S. aureus, L. monocytogenes*, *E. coli* and *B. cereus* in mango juices with low survival of injured cells during room temperature storage. (23) Similar antibacterial effects on reducing pathogen survival in other food samples have been observed by combining ZnO NPs with

acetic acid addition (24).

4. CONCLUSION

Using a combination of mild heat and ZnO NP treatments can successfully control *E. coli*, *L. monocytogenes* and *S. aureus* in the samples, and the combinations of heat and ZnO NP are synergistic. Mild heating plus ZnO NP treatment are synergistic, so combined treatments can be developed, which would decrease the temperature and amount of ZnO NP required for minimally processed milk during maintaining pathogen control.

ACKNOWLEDGMENT

This work was supported by Payame Noor University, Yazd, Iran. Authors gratefully acknowledge the generous cooperation of Nano Structured Coatings Institute, Yazd Payame Noor University, PO Code 89431-74559 Yazd, Iran.

AUTHORS CONTRIBUTION

This work was carried out in collaboration between all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

REFERENCES

1. Okouchi S, Murata R, Sugita H, Moriyoshi Y, Maeda N. Calorimetric evaluation of the antimicrobial activities of calcined dolomite. JOURNAL OF ANTIBACTERIAL AND ANTIFUNGAL AGENTS JAPAN. 1998;26:109-14.
2. Wilczynski M, editor. Anti-Microbial Porcelain Enamels. 62nd Porcelain Enamel Institute Technical Forum: Ceramic Engineering and Science Proceedings, Volume 21, Issue 5; 2000: Wiley Online Library.
3. Li Q, Mahendra S, Lyon DY, Brunet L, Liga MV, Li D, et al. Antimicrobial nanomaterials for water disinfection and microbial control: potential applications and implications. Water research. 2008;42(18):4591-602.
4. Yamamoto O. Influence of particle size on the antibacterial activity of zinc oxide. International Journal of Inorganic Materials. 2001;3(7):643-6.
5. Zhang L, Jiang Y, Ding Y, Povey M, York D. Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids). Journal of Nanoparticle Research. 2007;9(3):479-89.
6. Mirhosseini M, Firouzabadi FB. Preparation of ZnO-Polystyerne Composite Films and Investigation of Antibacterial Properties of ZnO-Polystyerne Composite Films. IRANIAN JOURNAL OF PATHOLOGY. 2014;9(2):99-106.
7. Seven O, Dindar B, Aydemir S, Metin D, Ozinel M, Icli S. Solar photocatalytic disinfection of a group of bacteria and fungi aqueous suspensions with TiO< sub> 2</sub>, ZnO and Sahara desert dust. Journal of Photochemistry and Photobiology A: Chemistry. 2004;165(1):103-7.
8. Brayner R, Ferrari-Iliou R, Brivois N, Djediat S, Benedetti MF, Fiévet F. Toxicological impact studies based on Escherichia coli bacteria in ultrafine ZnO nanoparticles colloidal medium. Nano Letters. 2006;6(4):866-70.
9. Sawai J. Quantitative evaluation of antibacterial activities of metallic oxide powders (ZnO, MgO and CaO) by conductimetric assay. Journal of microbiological methods. 2003;54(2):177-82.
10. Roselli M, Finamore A, Garaguso I, Britti MS, Mengheri E. Zinc oxide protects cultured enterocytes from the damage induced by Escherichia coli. J Nutr. 2003;133(12):4077-82.
11. Sandstead HH. Understanding zinc: recent observations and interpretations. The Journal of laboratory and clinical medicine. 1994;124(3):322-7.
12. Jones N, Ray B, Ranjit KT, Manna AC. Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms. FEMS microbiology letters. 2008;279(1):71-6.
13. Premanathan M, Karthikeyan K, Jeyasubramanian K, Manivannan G. Selective toxicity of ZnO nanoparticles toward Gram-positive bacteria and cancer cells by apoptosis through lipid peroxidation. Nanomedicine : nanotechnology, biology, and medicine. 2011;7(2):184-92.
14. Shin JH, Lee SY, Dougherty RH, Rasco B, Kang DH. Combined effect of mild heat and acetic acid treatment for inactivating Escherichia coli O157:H7, Listeria monocytogenes and Salmonella typhimurium in an asparagus puree. Journal of applied microbiology. 2006;101(5):1140-51.
15. Jin T, Sun D, Su JY, Zhang H, Sue HJ. Antimicrobial efficacy of zinc oxide quantum dots against Listeria monocytogenes, Salmonella Enteritidis, and Escherichia coli O157:H7. Journal of food science. 2009;74(1):M46-52.
16. Mirhosseini M, Firouzabadi FB. Antibacterial activity of zinc oxide nanoparticle suspensions on food-borne pathogens. International Journal of Dairy Technology. 2013;66(2):291-5.
17. Leistner L. Basic aspects of food preservation by hurdle technology. International Journal of Food Microbiology. 2000;55(1):181-6.
18. Cowan ST, Steel KJ, Barrow G, Feltham R. Cowan and Steel's manual for the identification of medical bacteria: Cambridge university press; 2003.
19. Huang Z, Zheng X, Yan D, Yin G, Liao X, Kang Y, et al. Toxicological effect of ZnO nanoparticles based on bacteria. Langmuir. 2008;24(8):4140-4.
20. Liu Y, He L, Mustapha A, Li H, Hu Z, Lin M. Antibacterial activities of zinc oxide nanoparticles against Escherichia coli O157: H7. Journal of applied microbiology. 2009;107(4):1193-201.
21. Juneja VK, Eblen BS. Predictive thermal inactivation model for Listeria monocytogenes with temperature, pH, NaCl, and sodium pyrophosphate as controlling factors. Journal of Food Protection®. 1999;62(9):986-93.
22. Anellis A, Lubas J, Rayman MM. HEAT RESISTANCE IN LIQUID EGGS OF SOME STRAINS OF THE GENUS SALMONELLA a, b. Journal of food science. 1954;19(1-6):377-95.
23. Firouzabadi FB, Noori M, Edalatpanah Y, Mirhosseini M. ZnO nanoparticle suspensions containing citric acid as antimicrobial to control< i> Listeria monocytogenes</i>,< i> Escherichia coli</i>,< i> Staphylococcus aureus</i> and< i> Bacillus cereus</i> in mango juice. Food Control. 2014;42:310-4.
24. Mirhosseini M, Arjmand V. Reducing Pathogens by Using Zinc Oxide Nanoparticles and Acetic Acid in Sheep Meat. Journal of Food Protection®. 2014;77(9):1599-604.