Original Article

Reduction of *Listeria monocytogenes* and *Bacillus cereus* in Milk by Zinc Oxide Nanoparticles

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ABSTRACT

Background & Objectives: **Direct addition of antimicrobial materials to food during food processing is an effective method for controlling microbial contaminants of food and extending the shelf- life of food products. Objective of this research was to study the antimicrobial effect of zinc oxide (ZnO) nanoparticle and potential applications of ZnO nanoparticles in terms of controling two food-borne pathogens in milk.**

Methods: **Toxicity of different concentration (0, 0.5, 2, 5, and 10 mM) of ZnO nanoparticles on *Listeria monocytogenes* and *Bacillus cereus* was studied in culture media and milk.**

Results: **Among the mentiond concentrations, treatment of 10 mM of ZnO nanoparticle was the most effective one for *L. monocytogenes* and *B. cereus* inhibition, which completely inhibited the growth of *L. monocytogenes* and *B. cereus* in 24h. These data revealed concentration-dependency of the antibacterial activity of ZnO. Therefore, 5 mM and 10 mM ZnO were selected for further studies, which were performed in milk, since they demonstrated significant growth inhibition. ZnO NPs were more capable in terms of reducing the initial growth counts of all the above-stated strains in milk.**

Conclusion: **ZnO nanoparticles had an antimicrobial activity against *L. monocytogenes* and *B. cereus* in milk and the media. This work was a preliminary study that provided a starting point for determining whether the use of ZnO nanoparticles had the potential for being applied in food preservation or not.**

***Keywords: Listeria monocytogenes*, *Bacillus*, Zinc Oxide, Nanoparticles**

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Introduction

Outbreak of food-borne pathogens such as *E. coli*, *Salmonella* spp. and *Listeria monocytogenes* in food safety has attracted the public attention to the need for developing new antimicrobial materials in order ensure food safety and extend its shelf- life (1, 2). Direct addition of antimicrobial materials to food or packaging materials during food processing is an effective method which could control microbial contaminants in food and extend the shelf-life of food products (3). Recently, inorganic antimicrobial materials, such as metal oxides, have been applied in various areas for controling microbes (1, 2).

Curently, most of the antimicrobial properties of minerals such as TiO_2 (4, 5) and ceramics immobilized by antimicrobial metals, such as silver and copper have been studied (1, 6, 7). Inclusion of metallic oxides in vitreous oxidic matrices has been a structural objective for obtaining compounds with improved physical, chemical, and biological properties that can be applied in medical fields.

It has been demonstrated that zinc oxide may induce different effects while it is used as a granule. The apply of metal and metallic oxides as antimicrobial materials has also the advantage that metal or metal ions are essential for human body (8).

Reduction of material to nano size changes their activity and properties, while improving their physical, chemical, and biological properties (9). There has been a report about the possibility of nanoparticle-based formulations for antimicrobial materials, which may themselves be effective in controlling the spreading new resistant strains of bacteria such as *Staphylocccus aureus* (10). Therefore, further research is necessary to develop new nanoparticle formulations which are stable under harsh processing conditions, and are also effective in destroying of microorganisms (3).

Owing to its strong antimicrobial effect on a board spectrum of microorganisms like *Escherichia coli* O157:H7, *Salmonella, Listeria monocytogenes*, *Staphylococcus aureus* (11) and *Lactobacillus* (12) ZnO has found many applications in daily life, which include drug delivery, cosmetics, and medical devices. Moreover, zinc oxide is one of the five zinc compounds that are currently listed as a substance generally recognized as safe (GRAS) by Food and Drug Administration of the United States of America (21CFR182.8991) (13, 14). Food industry uses ZnO as a source of zinc, that is an essential micronutrient for human body and animal cell, which are needed for more than 300 biochemical reactions in the body (11). In food industry, ZnO has been also applied as food additive, breakfast cereals, and animal feedstuff (3, 11). However, few information exists about the antimicrobial efficacy of ZnO in food.

In the present work, the antibacterial activity, of ZnO nanoparticle powder was studied in culture media and milk. The objective of this study was to examine the antimicrobial effect of ZnO nanoparticle and its potential applications in terms of controling two food-borne pathogens in milk.

Materials and Methods

Bacterial strains, media, and materials

The following bacterial strains were applied in this reserach: *L. monocytogenes* PTCC1163 and *B. cereus* PTCC1015 were obtained from the culture collection of I.R. Dept. Stock cultures were maintained at −80 °C. These strains were propagated on Tryptic Soy Agar (TSA: Merck, Germany) at 37 °C and maintained at 0 to 2 °C until use. ZnO nanoparticles were purchased from TECONAN, Spain (with particle diameters of 20–25 nm; and purity of 99.98%). They were dissolved in sterile double-distilled water and sonicated until being dispersed and forming a uniform colloidal suspension. All the experiments were caried out using a freshly prepared colloidal suspension of ZnO nanoparticle.

Determining antibacterial activity of ZnO nanoparticle

Spot on the lawn method

To test the antibacterial activity of ZnO nanoparticles, spot on the lawn method was employed. Antibacterial activity of ZnO nanoparticle was tested by spotting 20 µl of the ZnO nanoparticle solution (0, 0.5, 2, 5 and 10 mM) onto soft agar lawn (0.6%) seeded with 10^7 cell/ml *L. monocytogenes* and *B. cereus*, respectively. Each concentration of ZnO nanoparticle was placed on surface-inoculated TSA agars and incubated at 37 °C for 24h. The inhibition zone around each sample was applied to represent the antibacterial activity of each ZnO nanoparticle's concentration (15).

Detection Inhibitory Activity

For detecting the antibacterial and pathogenic-growth inhibitory activity of ZnO NPs, the Tryptic Soy Broth culture, (TSB; Merck, Germany), which contained 0, 0.5, 2, 5, and 10 mM NP, was inoculated with 10^7 cells/ml of *L. monocytogenes* and *B. cereus*, respectively. The bottles were shaken at the speed of 50 rpm at 37 °C. Then, optical density (OD) of the cultures was serially monitored every 2 h for up to 10–12 at 600 nm, the final reading was performed in 24 h. The cultured strains were used as the control without any NP formulation and under the same growth conditions. To avoid potential optical interference during the optical measurements of the growing cultures caused by the light scattering properties of NPs, the same liquid medium, which lacked any microorganisms but contained the identical concentration of NP, was cultured under similar conditions as blank controls (9).

Applying ZnO nanoparticles in milk

Two 5 and 10 mM concentrations of ZnO nanoparticle were selected as the antimicrobial treatments in milk samples. Two experimental groups of milk were separately added with the concentrations of 5 and 10 mM NP. Afterward, they were inoculated with 10^7 cells/ml concentration of *L. monocytogenes*, and *B. cereus*, respectively. The bottles were then incubated in an orbital shaker at the speed of 50 rpm at 25 °C for 24 h. Following the inoculation, the number of bacterial cells was determined every 4 h, for up to 24 h using agar count plate method (16).

Statistical Analysis

Antimicrobial experiments were conducted in triplicate. Data points were expressed as mean ± SD. Data were analysed by Analysis of Variance (ANOVA) in SAS software. Duncan's multiple range tests were applied to specify the significant difference between mean values. Except in the mentioned cases, 5% significance level was considered throut the study.

Results

Antibacterial activity in microbial culture media

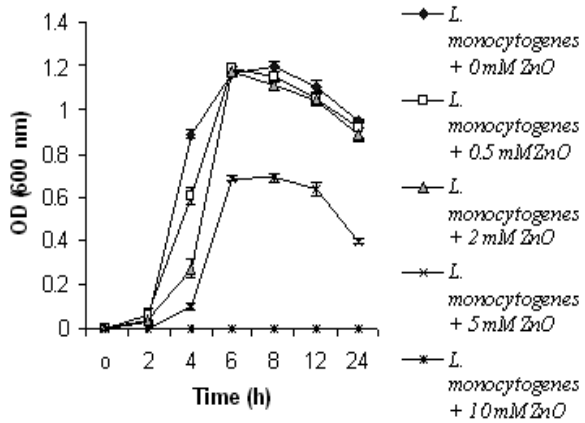
Antibacterial properties of 0, 0.5, 2, 5, and 10 mM ZnO nanoparticle were measured using inhibition zone method against *L. monocytogenes* and *B. cereus*.Table 1 demonstrates the results of inhibition zone of different concentrations of ZnO, representing that 0, 0.5 and 2 mM ZnO had no inhibition zone on *L. monocytogenes* (P>0.05) and 5 and 10 mM ZnO had 9±0.3 and 10±0.1 mm inhibition zone against *L. monocytogenes*, respectevily (P<0.05). As can be seen, inhibition area increased once with the molar content of ZnO. Moreover, results demonstrated that 0, 0.5, 2 and 5 mM concentration of ZnO had no inhibition zone against *B. cereus* (P>0.05). Further, sensitivity of *L. monocytogenes* was found to be larger than that of *B. cereus* in 24h.

Table 1- Inhibition zone diameter different Concentration of ZnO.

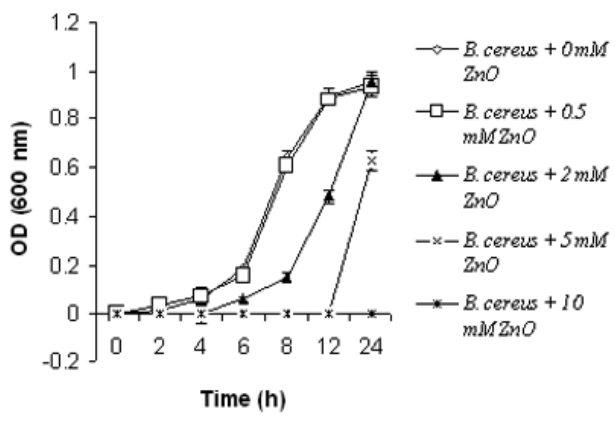
Concentration of ZnO (mM)	*L. monocytogenes (mm)*	*B. cereus (mm)*
0	-*	-
0.5	-	-
2	Gr**	-
5	9±0.3	-
10	10±0.1	8±0.1

*No inhibition zone **Growth reduction

Figure 1 shows the effect of ZnO nanoparticle treatment on the growth of *L. monocytogenes* and *B. cereus* in TSB broth at 37 ◦C. Treatments with the ZnO concentration of 0.5, 2, 5 and 10 mM had a significant inhibitory effect on the growth of *L. monocytogenes* and *B. cereus* during 24h of incubation, compared to the control. Among the four concentrations of ZnO nanoparticles, the treatment with 10mM ZnO nanoparticle was the most effective one for *L. monocytogenes* and *B. cereus* inhibition, which completely inhibited the growth of *L. monocytogenes* and *B. cereus* in 24h (P<0.05). Also, results demonstrated that 0.5 and 2 mM ZnO nanoparticle had no inhibitory effect on *L. monocytogenes* and *B. cereus* in 24h in TSB (P>0.05). Moreover, 5 mM ZnO nanoparticle had an inhibitory effect on *L. monocytogenes* and *B. cereus* in TSB. OD of *L. monocytogenes* in TSB with 5mM ZnO nanoparticle was 0.691 in 8h, 0.641 in 12h, and 0.398 in 24 h, while the control values were 1.192, 1.105, and 0.945, respectively. Results represented that 5mM ZnO nanoparticle caused 57.89% growth reduction in *L. monocytogenes* after 24h (P<0.05). Also, OD of *B. cereus* in TSB with 5mM ZnO was 0 in 12h, and 0.626 in 24h while the control values were 0.643 in 8h, 0.902 in 12h, and 0.956 in 24h. These data showed that 5mM ZnO completely inhibited the growth of *B. cereus* in TSB in 12h; however, it caused 34.52% growth reduction of *B. cereus* in 24h (P<0.05). ZnO nanoparticle showed strongest antibacterial activity against *L. monocytogenes* at 24h. These data revealed that the antibacterial activity of ZnO nanoparticle was concentration- dependent, as was already approved using agar diffusion method.



a

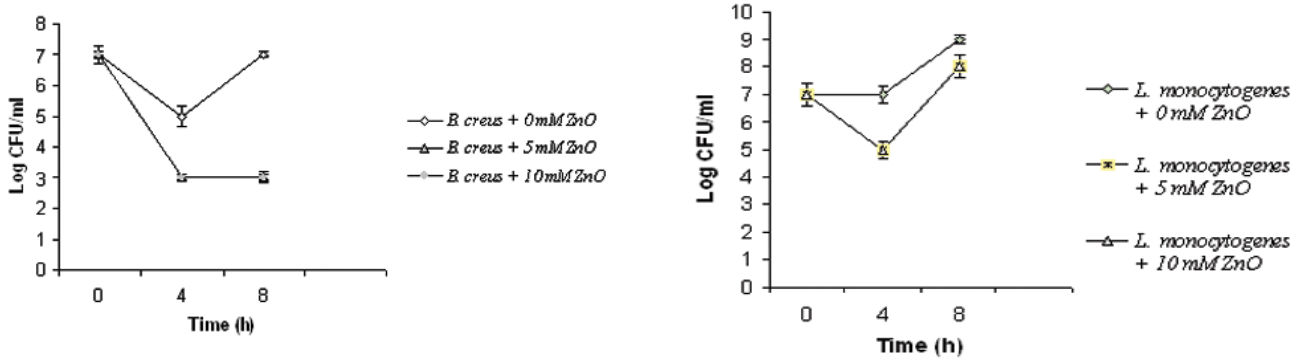


b

Fig.1: Effect (0, 0.5, 2, 5 and 10) mM ZnO on growth of **(a)** *L. monocytogenes* and **(b)** *B. cereus* in TSB at 37 ◦C

Applying ZnO Nanoparticles in Milk

Two 5 and 10 mM concentrations of ZnO nanoparticle were used as antimicrobial treatments in the milk samples. Effect of ZnO nanoparticle on the growth of *B. cereus* in milk during 8 h of storage at 25 °C is demonstrated in Figure 2. After an initial drop from 7 log CFU/mL in 8 h, *B. cereus* cells in the control remained at 7 log CFU/mL, while the cells in the milk sample which were treated with 5 and 10 mM of ZnO nanoparticle were reduced to 3 log CFU/mL. Results showed that ZnO nanoparticles could significantly inhibit or reduce *B. cereus* in milk ($P<0.05$). Moreover, Figure 2 demonstrates that the antibacterial effect of ZnO nanoparticle on *B. cereus* was stronger than that on *L. monocytogenes*. These findings implied that the treatment efficacy was more similar to the one obtained by ZnO nanoparticle in the first set of experiments (Fig.1).



a b

Fig. 2: Effect (5 and 10) mM ZnO on growth of (a) *B. cereus* and (b) *L. monocytogenes* in milk at 25 °C

Discussion

Nanotechnology has found many applications in important areas of science, which include food security, targeted treatment of diseases, new tools for cellular and molecular biology, new materials for pathogen detection, and environmental protection (17). Nanoparticles are applied in nano sensors and nanotracers (18).

In this study, antibacterial activity of ZnO nanoparticle was studied in cultur media and milk. Treatments including the antibacterial effect of ZnO NP suspension were surprisingly significant in terms of reducing the cell count and surviving of *L. monocytogenes* and *B. cereus*. The present results showed that ZnO NP had a better inhibitory effect on all the experimental strains, when cultured in a liquid medium, rather than the solid culture. Thus, 5 and 10 mM of ZnO were selected for further studies in milk, in which, significant growth inhibition was found in all bacterial strains, when cultured in TSB (Fig. 1).

Figure 2 demonstrates the log-reduction of all strains, inoculated onto milk, which were treated with different 0,5 and 10 mM concentrations of ZnO suspensions. Results exhibited that ZnO had an inhibitory effects on the growth of all strains in milk, during the culturing period of 8 h, as compared to the control, which was further confirmed in the media culture.

When ZnO powders were applied, ZnO particles were observed at the bottom of the bottles during the incubation, which was due to the deposition of ZnO. Deposition of ZnO was probably the reason for the decreased antibacterial activity. Based on the above mentioned results antimicrobial ZnO molecules are required to be in contact with the cell and enter the cells. Mechanism of the growth inhibition of ZnO in culture media may originate

from killing or growth suppression. At higher concentrations of ZnO, ZnO treatments first killed the sensitive sub-population of cells and reduced the microbial population. Afterward, they returned the growth by a resistant ZnO subpopulation. At lower concentrations of ZnO (Fig.1), ZnO treatments only suppresed the growth of bacteria and caused the 4 or 6 h delay in the growth of the tested bacteria.

For a long time, ZnO powder has been used as an active ingredient with antibacterial properties in skin creams, lotions, and ointments (19, 20). However, zinc oxide nanoparticles are more effective in controlling the growth of microorganisms; furthermore, initial studies have shown that smaller particles have a higher inhibitory effect on bacterial growth. Photo-activated (e.g. by UV light) TiO_2 has been used to eliminate various bacteria including MRSA (21, 22), however, non-activated TiO_2 did not significantly inhibit the growth of *S. aureus*. Studies on the toxicity of ZnO nanoparticles on *E. coli* were performed by plate assays and transmission electron microscopy analyses. Such studies demonstrated that synthesized ZnO nanoparticles could slow down the bacterial growth. Result demonstrated that the disorganization of *E. coli* membranes, increased the membrane permeability and thus led to the accumulation of nanoparticles in the bacterial membrane and cytoplasm regions of the cells (23).

At present, very few reports are available about the use of nanoparticles in food preservation. For example, zinc oxide (ZnO) quantum dots are used as antimicrobial agents in liquid egg white samples. Depending on their concentration, ZnO quantum dots could significantly inhibit or reduce *L. monocytogenes* and *S. enteritidis* in liquid egg white (16). Similar inhibitory effects of ZnO NPs on reducing *S. aureus* and *E. coli* have been observed in milk samples (24).

ZnO nanoparticles are believed to destruct the lipids and proteins of the bacterial cell membrane, and therefore result in the leakage of intracellular contents and death of bacterial cells (25, 26). In addition, production of hydrogen peroxide and Zn^{+2} ions has been proposed as the key antibacterial mechanisms in ZnO nanoparticles (19).

Conclusion

ZnO nanoparticles have an antimicrobial activity against *L. monocytogenes* and *B. cereus* in milk and the media. According to this study, inhibitory effect of zinc oxide nanoparticle was concentration-dependent. The higher concentration of the applied ZnO nanoparticle, the higher the antibacterial effect would be. This work can be considered a preliminary study that provided a starting point for determining whether the use of ZnO nanoparticles had the potential application in food preservation or not. Further research is needed to determine the optimal parameters of antimicrobial activity. Parameters including concentration, time, temperature, and combination with other bacteriocins (synergistic effect) could be the focus of future works.

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