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Antibacterial Efficacy of Zinc Oxide and Titanium Dioxide Nanoparticles against *Escherichia coli* in Minced Meat

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ABSTRACT

Antibacterial nanoparticles are a new approach to control the safety of meat and meat products. This work aimed to investigate the antibacterial effect of zinc oxide (ZnO) and titanium dioxide (TiO_2) nanoparticles, alone or together, against *Escherichia coli*. Antibacterial activity of these nanomaterials was evaluated using the disc diffusion method. In this regard, minced meat samples were inoculated with *E. coli* and treated with different concentrations of two nanomaterials (approximately 20 nm), including 6 mM and 12 mM ZnO, 6 mM and 12 mM TiO2, and a combination of 6 mM ZnO and 6 mM TiO2, then stored at 4°C for 17 days. The results indicated that ZnO (12 mM) had a significant reduction effect on *E. coli* count in minced meat, followed by the combination of ZnO and TiO2, and 12 mM TiO2 alone. The antibacterial activity of ZnO, TiO2, and combination of ZnO and TiO2 was also examined using a transmission electron microscope and it was found that 12Mm ZnO had a higher destructive effect on bacterial cell than the mixture of ZnO + TiO2, and 12Mm TiO2 alone. The disc diffusion method showed that ZnO (12 mM) was the most effective concentration used against *E. coli*. It is concluded that 12 mM ZnO nanoparticles have the best antibacterial effect against *E. coli* in minced meat stored at 4 °C for 17 days.

Keywords: *E. coli*, Minced meat, Nanoparticles, TEM, Titanium dioxide, Zinc oxide

INTRODUCTION

Increasing meat consumption throughout the world presents excessive challenges to meat safety and hygiene (Sofos and Geornaras, 2010). Meat product deterioration during distribution and exposure through the markets has negative effects on the meat industry from an economic point of view (Domínguez et al., 2018). Ground meat not only is highly susceptible to spoilage, but also is frequently involved in the spread of pathogens (Ahmed and Ismail, 2010) especially pathogenic strains of *E. coli* (Marcous et al., 2017), therefore, it is necessary to search for substances that are effective against bacteria especially *E. coli* and improve the microbiological condition of ground meat. Source tracking of *E. coli* is the main step to control foodborne infections (Liu et al., 2019). Great efforts are being made in the food industry for improving hygiene and increasing the shelf life of meat products through preventing the growth and multiplication of food-borne pathogens (Baltić et al., 2013).

Nanotechnology can provide the method that can be applied throughout different aspects of the food chain processing to improve food safety and quality control and increase food shelf life (Baltić et al., 2013). The most important nanomaterials that commonly used for antibacterial activity in the food industry are oxides of zinc (Zn) and titanium (Ti) (Duncan, 2011). Titanium dioxide (TiO_2) and zinc oxide (ZnO) nanoparticles are known to be one type of inorganic multifunctional substances that are able to inhibit the growth of microbes and they have been listed as Generally Recognized As Safe (GRAS) by the U.S. FDA (Zambrano-Zaragoza et al., 2018). These nanoparticles act as biocides and do not have any toxic effect and have approved by FDA for application in food processing fields (Toker et al., 2013). TiO2 is commonly used as a food additive and authorized for use in the European Union as E171 (Directive, 1994).

It is supposed that the toxicity of nanoparticles depends on their morphology, size, and amount consumed. Although possible risk to human health after eating food having TiO2 nanoparticles has been poorly explored, scientific databases inform that TiO2 nanoparticles can induce inflammation due to oxidative stress and also can have a genotoxic effect leading to chromosomal instability (Baranowska et al., 2020).

The antibacterial effect of TiO_2 and ZnO nanoparticles against *E. coli* O157:H7 in fresh calf minced meat have been investigated (Marcous et al., 2017). The antibacterial activity of TiO_2 nanoparticle against *E. coli* was investigated by Othman et al. (2014), who proved the importance of TiO2 nanoparticles for ensuring the safety of food.

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One of the most important ways for applications of nanomaterials in food and meat is to place nanoparticles directly into food as food additives (Coles and Frewer, 2013) to preserve colors and prevent spoilage (Xie et al., 2011). Most previous studies link nanoparticles to external coating or packaging of meat but these methods are not appropriate for use in the minced meat industry. In addition, EFSA (2008) and Avella et al. (2005) supposed that migration of nanoparticle from packaging materials is either nil or very low, therefore, this study aimed to examine the antibacterial effect of ZnO and TiO2 nanoparticles, alone or together, directly mixed with minced meat. Also, the present study evaluated the *in vitro* antimicrobial effect of these nanomaterials against *E. coli.*

MATERIALS AND METHODS

The experiment was conducted in the Animal Health Research Institute, Egypt.

Minced beef

Fresh minced beef used in this study was purchased and immediately transported to the laboratory in an icebox and stored at 4 °C until use. Thin sheets of minced beef were treated with ultraviolet light (wavelength 385 nm) for 30 min, 15 min to each side to eliminate background microflora (Morsy et al., 2018).

Bacterial strain

Escherichia coli (ATCC® 25922™) ~ 8 log CFU/ ml was used in this study and obtained from Media Unit, Food Hygiene Department, Animal Health Research Institute, Dokki, Giza, Egypt.

Synthesis and preparation of zinc oxide nanoparticles

Zinc oxide nanoparticles were prepared by dissolving 11 g zinc acetate hydrate with 99.9% purity ($Zn(Ac)_2$•2H2O, Sigma-Aldrich) in 500 ml ethanol. Then, 2.9 g sodium hydroxide was added into the solution through ultra-sonication, and a transparent solution was obtained. The conical flask containing the transparent solution was put into a water tank with a constant temperature of 60 °C. After that, 10 ml of distilled water was added to the solution into the conical flask. The solution was stirred for 30 min at 60 °C. The prepared ZnO nanoparticles were collected by centrifuging and drying at 60 °C (Wang et al., 2007).

Synthesis and preparation of titanium dioxide nanoparticles

Titanium tetrachloride ($TiCl_4$) (Fluka 98%) was used as a starting material. TiO2 nanoparticles were prepared by dropwise addition of 4 ml of $TiCl_4$ into 400 ml of water/ethanol solution (3:1) at 0 °C with vigorous stirring. Subsequently, a dilute solution of NH_4OH was used to adjust the pH at 9. The solution was refluxed for 4 h with continues stirring. Then the solution was cooled down to room temperature naturally. The TiO2 nanoparticles were obtained by centrifuging at 4000 rpm. The formed TiO2 was washed using acetone several times and then dried at 100 °C for 5 h. The powder was annealed at 400 °C in air for 2 h by raising the temperature at a rate of 10 °C/min (Yin et al., 2001).

Assessment of *in vitro* antibacterial activity of nanomaterials using disc diffusion method

To assess the inhibitory range of nanoparticles of ZnO (2, 3, 5, 6, and 12mM) and TiO2 (3, 4, 5, 6, and 12mM) against *E. coli*, they were suspended in double-distilled water and constantly stirred until a uniform colloidal suspension was formed to yield solutions of different concentrations. An appropriate volume of test bacteria was inoculated on Mueller-Hinton agar medium. Sterile paper discs (Whatman No.1, 6 mm in diameter) were placed on the surface of suitable media plates freshly inoculated with bacterial cells, then 10µL from each dilution was dispensed onto the surface of each disc. Plates were then incubated for 24 h at 37 °C (Bauer et al., 1966).

Assessment of antibacterial activity of nanomaterials in minced meat

In a sterile bag, minced meat was inoculated with *E. coli* (~ 8 log CFU/ml) to achieve final concentration ~ 6 log CFU/g of minced meat. Then, they were mixed thoroughly by gently squeezing the bags by hand till even distribution of microbe occurred, and left for 30 min for complete attachment between inoculated *E. coli* and minced meat. The initial load of *E. coli* was determined before the addition of nanomaterials. Phosphate buffer saline (PBS) was used for the treatment of control samples. Minced meat sample was divided into six groups (200 g each); Group 1 (PBS + *E. coli*), Group 2 (6mM ZnO + *E. coli*), Group 3 (6mM TiO2 + *E. coli*), Group 4 (12mM ZnO + *E. coli)*, Group 5 (12mM TiO2 + *E. coli*), and Group 6 (6mM ZnO + 6mM TiO2 + *E. coli*). Nanomaterials were mixed with the minced beef samples for a further 30 seconds to ensure even mixing. All samples were transferred individually into a standard sterile polyethylene bag (self-closed). Packed samples were labeled and kept at 4 ± 1 °C till spoilage of minced meat. Counting of *E. coli* and sensory evaluation were performed on days 0, 3, 6, 9, 12, 15, and 17. The experiment was repeated in triplicate for each group and mean values were calculated.

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E. coli enumeration

Accurately, 100 µl from each previously prepared serial dilution was spread over duplicated plates of EMB agar (OXOID, CM0 069) using a sterile bent glass spreader. The inoculated and control plates were incubated at 37 °C for 24 h (FDA, 2001). The suspected colonies of *E. coli* were greenish metallic colonies with a dark purple center. These colonies were enumerated and expressed as log CFU/g of sample.

Electron microscopy observations

Transmission Electron Microscopy (TEM) techniques used to evaluate biocidal action of nanomaterials on *E. coli* using TEM Negative Staining method**.** JEOL JEM1400 transmission electron microscope was used (Yashroy, 1990). TEM was conducted in Cairo University Research Park, Egypt.

Sensory evaluation

Sensory evaluation was performed under the controlled condition of temperature (28 °c), humidity (65%), and light by five well-trained female panelists of 30 to 35 years of age, who were selected according to ISO (2012). The panelists were able to perform descriptive sensory analysis for treated samples and control one and give reliable comparative judgments. The criteria used as the basis of the organoleptic descriptive assessment and the samples were rated on a continuous hedonic scale (ISO, 2003). The panel received a list of descriptors (odor, color, and texture) to score on numerical and continuous scales from 0 (the lowest score for each attribute, very bad) to 10 (the highest score for each attribute, very good) according to Cullere et al. (2018). Every one of panelists took disposable dish containing three samples (two identical and another different) in triangle form randomly coded with four numbers and worksheet to give the score for each point. A mean score of lower than 5 indicated unacceptable quality. Totally, 210 samples (50 g) were examined on 7 sessions, 30 samples per session on five rounds per session at 1st day, 3rd day, 6th day, 9th day, 12th day, 15th day and 17th day.

Statistical analysis

The experiment was designed in completely randomized design in a 6×7 factorial design; 6 treatments (6 mM ZnO, 12 mM ZnO, 6 mM TiO2, 12 mM TiO2, 6 mM of ZnO + TiO2 and control one) during 7 sampling days (1st day, 3rd, 6th, 9th, 12th, 15th and 17th) at refrigerated storage (4±1 °C). The experimental model was made according to Butler et al. (2009). All data were subjected to analysis of variance (ANOVA) using SPSS program for Windows (Version 22) (SPSS Inc. Chicago, IL, USA). F-values at the $p \leq 0.05$ were indicated significantly different. Duncan's multiple range test was used for measuring the specific differences between pairs of means (Duncan, 1955). Values presented as the means ± standard error

RESULTS AND DISCUSSION

In vitro antibacterial activity of nanomaterials using disc diffusion method

As shown in table 1, zones of inhibition were differed according to the concentration of nanoparticles used. Results showed that 12 mM ZnO had the widest inhibition zone, followed by 12mM TiO2, 6 mM ZnO, and 6 mM TiO2. While 2mM ZnO and 3 mM TiO2 do not show any antibacterial effect. According to a study, 3 mM and 6 mM concentrations of ZnO nanoparticles resulted in less bacterial growth compared to the control, while the growth of *E. coli* O157:H7 was completely inhibited by 12 mM ZnO nanoparticles (Liu et al., 2009). The antibacterial action of ZnO nanoparticle was studied by Emami-Karvani and Chehrazi (2011) against Gram-negative bacteria (*E. coli*) were used as test microorganisms. It was found that the antibacterial activity of Zn O nanoparticles increased with decreasing particle size ~20 nanometers and increasing powder concentration 12 mM. These small concentrations were able to prevent the growth of *E. coli* on its media so we used it in our challenge study.

Table 1. Antibacterial activity assessment of nanoparticles against *E. coli* using disc diffusion method

Nanoparticle (concentration)	Zone of inhibition(mm)
ZnO (2mM)	ND*
ZnO (3mM)	6 ± 0.12
ZnO (5mM)	7.5 ± 0.11
ZnO (6mM)	10 ± 0.10
ZnO (12mM)	15 ± 0.14
TiO_2 (3mM)	ND*
TiO_2 (4mM)	6 ± 0.02
TiO_2 (5mM)	7 ± 0.025
TiO_2 (6mM)	8 ±0.22
TiO_2 (12mM)	13 ±0.11

ND*: Not detected

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Electron microscopy observations on nanoparticle against *E. coli*

Figure 1 shows normal *E. coli* with an intact cell membrane (control one). As shown in figure 2, ZnO had the largest effect on bacterial cell as it affects the cell wall of bacteria so it becomes irregular, perforated, swelling, and over enlargement. Figure 3 shows the accumulation of nanoparticles (ZnO and TiO2) around the bacterial surface and agglomeration of cytoplasmic material. These results were in agreement with the findings of Ashe (2011), who reported ZnO nanoparticles are bactericidal and disrupt membrane thus cause membrane dysfunction, resulting in leakage the content outside and cell death. There are components found in Gram-negative bacteria, and not in Gram-positives, which can oppose nanoparticles to attachment onto cell walls; the possible mechanism is the extra layer of outer membranes and the pathogen-associated molecular patterns which include lipopolysaccharide and particular fragments of peptidoglycan. Bacterial cell wall properties can play a crucial role in the diffusion of nanoparticles inside the bacterial cell (Espitia et al., 2012). Figure 4 shows the effect of TiO2 on *E.coli* was less affected by TiO2 compared to ZnO, which showed over swelling and thinning of cell wall. The antibacterial effect of TiO_2 on some bacteria such as *E. coli* has been studied (Mihaly et al., 2015).

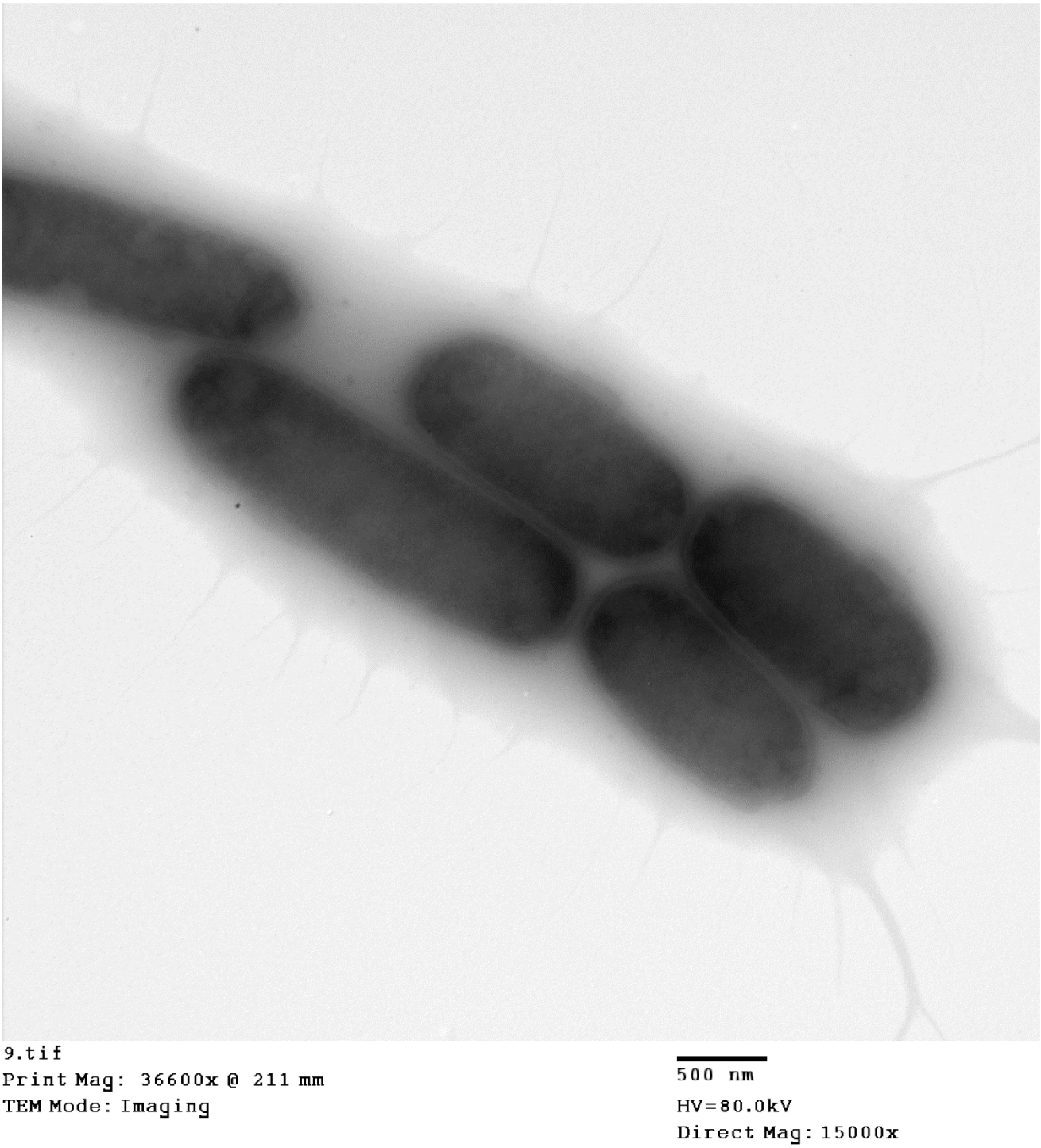

Figure 1. Normal morphology of *E. coli* using transmission electron microscope

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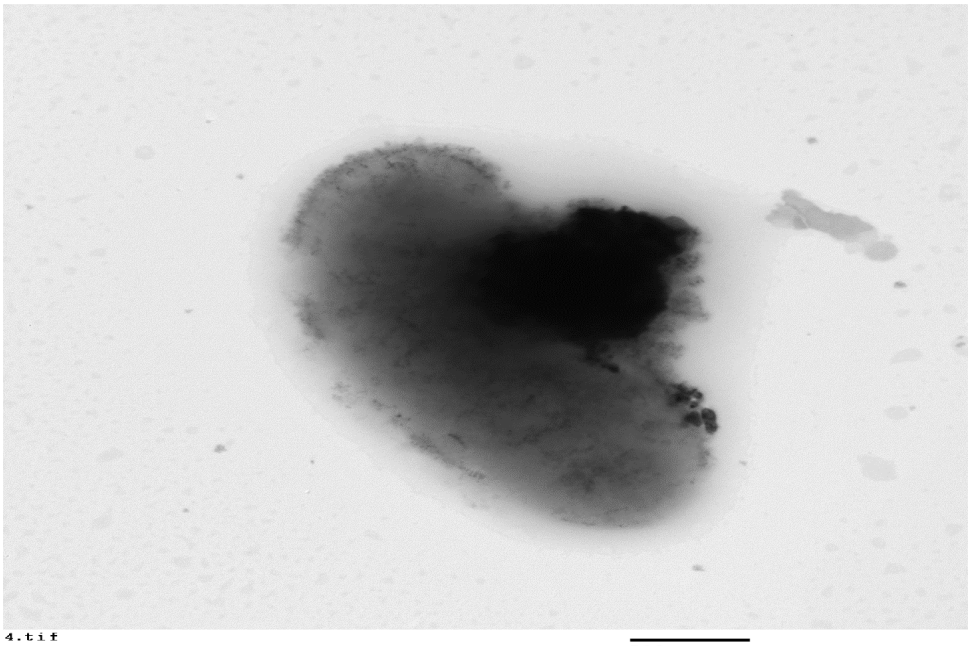

Figure 2. Antibacterial activity of ZnO nanoparticles against *E. coli* evaluated by transmission electron microscopy. ZnO nanoparticles adhered to *E. coli* cause pores in cell wall, elongation of cell, and ruptured cell.

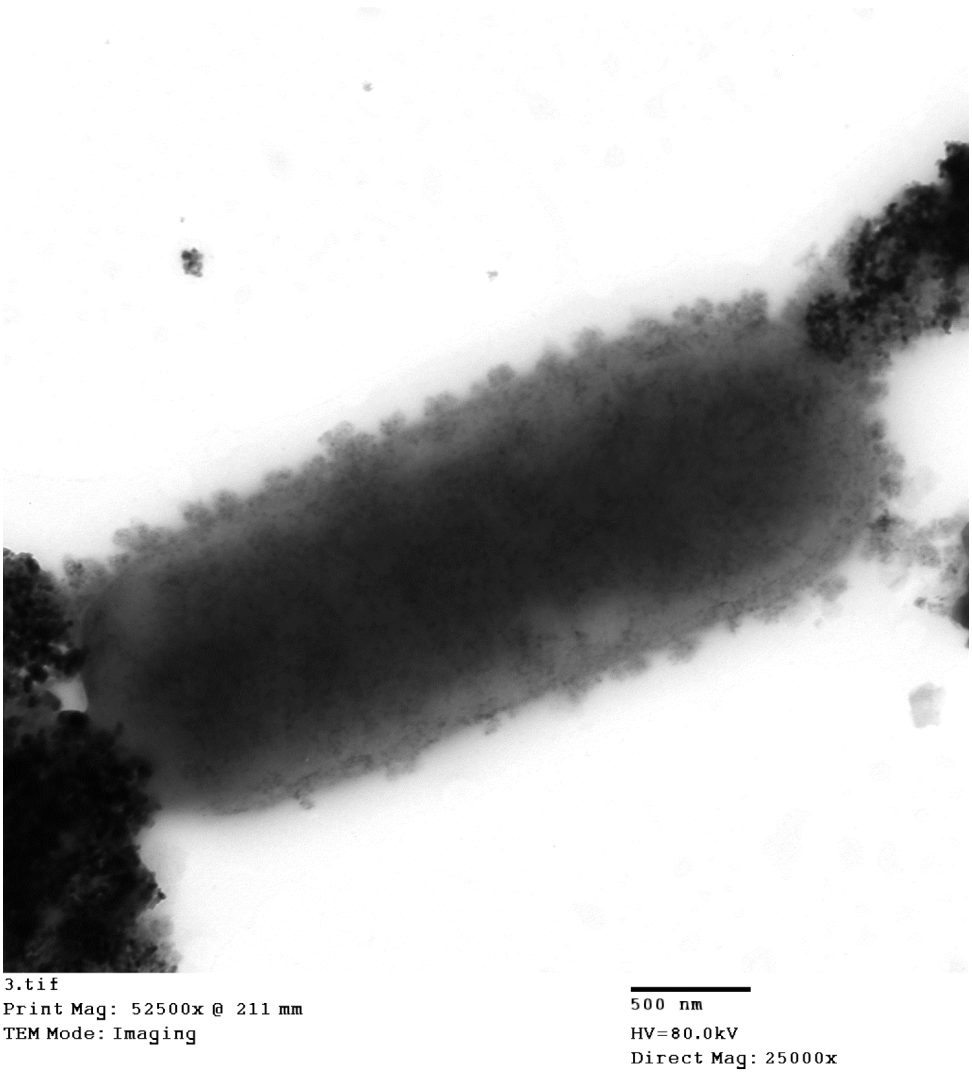

Figure 3. Antibacterial activity of nanoparticles mixture (ZnO + TiO2) against *E. coli* evaluated by transmission electron microscopy. Nanoparticles accumulated around the bacterial cell cause elongation of cell and disruption of cytoplasm.

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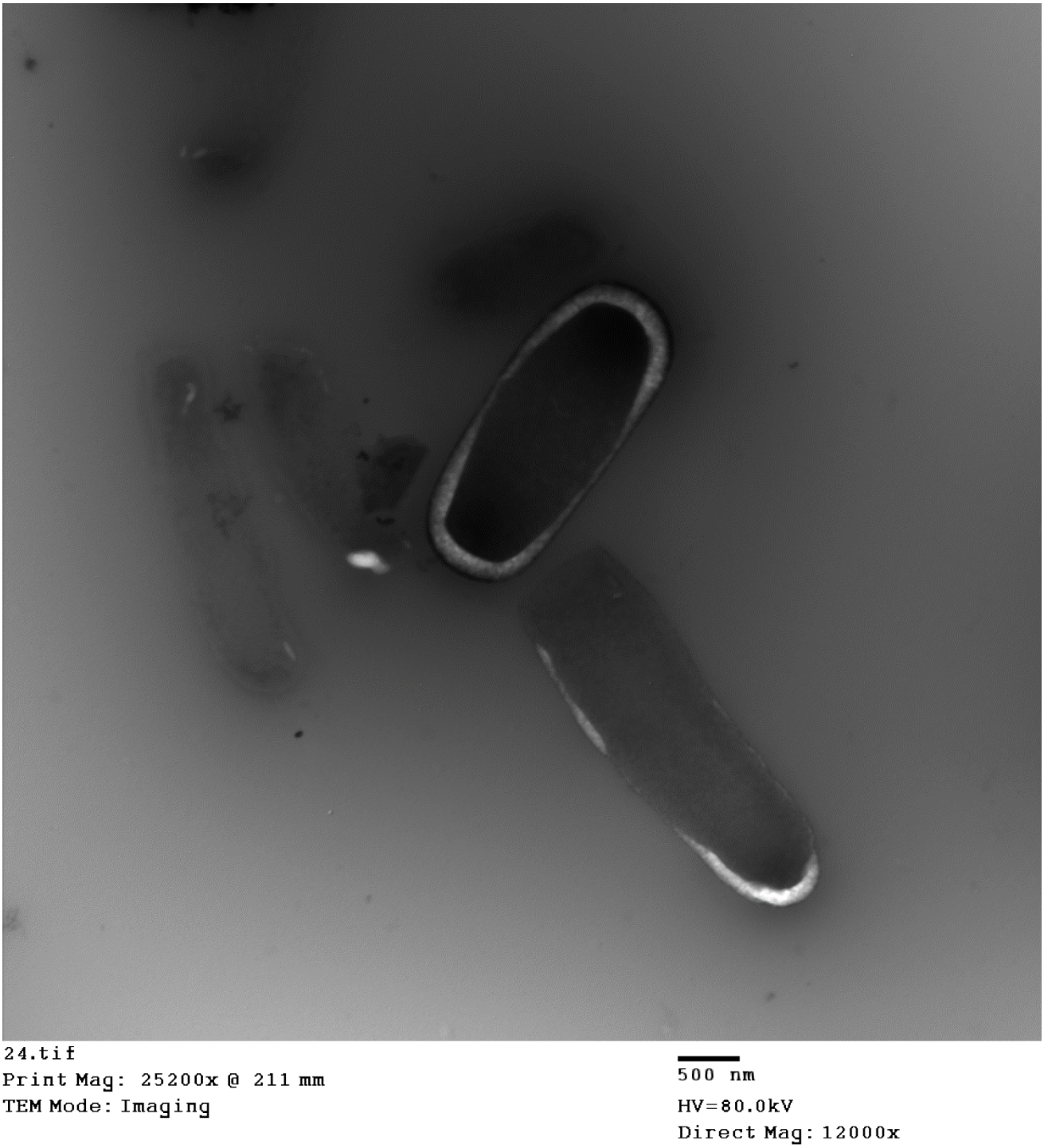

Figure 4. Antibacterial activity of TiO2 nanoparticles against *E. coli* evaluated by transmission electron microscopy. TiO2 caused elongation of cell, thinning of cell wall and pores in plasma membrane

***E. coli* count in minced meat stored at 4 °C**

The effect of nanoparticles on the count of inoculated *E. coli* in minced meat during storage at 4 °C is presented in table 2. *E. coli* counts increased in the control sample during the storage period of minced meat by ~ 2-4 log (CFU/g), which was significantly different from all treated samples. Count of *E. coli* in nanoparticles treated samples decreased throughout storage, indicating antibacterial activity of nanoparticles. ZnO (12 mM) exhibited great antibacterial effect against *E. coli* and it decreased the count by ~ 5 log (CFU/g) which was significantly different from the effects of obtained by the mixture of ZnO +TiO2 (6 mM) and TiO2 (12 mM) which decreased the count by ~ 4 log (CFU/g). Also, ZnO (6 mM) and TiO2 (6 mM) decreased the count by ~ 2log (CFU/g). These results are nearly similar to the findings of Marcous et al. (2017) who examined the antibacterial action of ZnO and TiO2, alone and together, against *E. coli* in calf minced meat and reported that ZnO was the most effective antimicrobial nanoparticles. ZnO nanoparticles are a novel material controlling foodborne pathogens, thus can be applied for food safety (Ali et al., 2020). Also, Morsy et al. (2018) studied the synergistic antimicrobial effect of ZnO nanoparticle and other compounds as nisin, lysozyme and EDTA nanoparticles on different foodborne pathogens including *E. coli* O157:H7 and proved that ZnO has great antimicrobial

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effect. As ZnO nanoparticles have great antibacterial activity, it has received significant interest worldwide particularly by the implementation of nanotechnology. Reduction of the particle size of ZnO nanoparticles leads to an increase in the particle surface reaction thus it exhibits great antibacterial activity (Sirelkhatim et al., 2015). Many studies investigated the effect of ZnO and TiO2 nanoparticles on *E. coli* at concentrations near concentrations used in this study but a very small diameter used in this study enhanced the effect of these nanoparticles. The concentrations of nanoparticles used in this study were less than the permissible limits approved by FDA (2015). ZnO and TiO2 nanoparticles are cheap antibacterial substances that have a wide range of antibacterial activity against microbes present in meat, therefore, they help to ensure the quality of meat, increase the shelf life for minced meat, and maintain the health of human.

Table 2. Antibacterial activity of different concentrations of ZnO and TiO2 nanoparticles against *E. coli* counts on minced beef inoculated with *E. coli* (~ 6 log CFU/g of minced meat) during storage at 4 °C for 17 days

Groups	1st day	3rd day	6th day	9th day	12th day	15th day	17th day
Control group	6.41 ± 0.26 ^{a}	6.28 ± 0.22 ^{a}	7.14 ± 0.1 ^{a}	8.64 ± 0.32 ^{a}	8.95 ± 0.6 ^{a}	8.99 ± 0.73 ^{a}	9.22 ± 0.14 ^{a}
12 mM ZnO	6.03 ± 0.6 ^{a}	5.38 ± 0.2 ^{c}	5.35 ± 0.1 ^{d}	4.78 ± 0.21 ^{c,d}	3.60 ± 0.2 ^{d}	1.65 ± 0.14 ^{c}	1.31 ± 0.9 ^{e}
6 mM ZnO + 6 mM TiO2	6.16 ± 0.7 ^{a}	5.55 ± 0.3 ^{c}	5.21 ± 0.1 ^{d}	4.33 ± 0.19 ^{d}	4.47 ± 0.3 ^{c}	2.11 ± 0.3 ^{c}	2.02 ± 0.10 ^{d}
12 mM TiO2	6.38 ± 0.8 ^{a}	5.63 ± 0.1 ^{b,c}	5.32 ± 0.2 ^{e}	4.90 ± 0.3 ^{b,c}	4.60 ± 0.23 ^{c}	3.02 ± 0.7 ^{d}	2.92 ± 0.10 ^{c}
6 Mm ZnO	6.40 ± 0.6 ^{a}	5.56 ± 0.09 ^{c}	5.43 ± 0.1 ^{c}	5.40 ± 0.6 ^{b}	4.71 ± 0.1 ^{b}	4.61 ± 0.68 ^{b}	4.30 ± 0.19 ^{b}
6 mM TiO2	6.40 ± 0.9 ^{a}	5.93 ± 0.1 ^{b}	5.51 ± 0.1 ^{b}	5.14 ± 0.5 ^{b}	4.94 ± 0.3 ^{b}	4.85 ± 0.73 ^{b}	4.27 ± 0.24 ^{b}

The values are expressed as Mean ± standard error of three experiments. Means within a column and rows followed by different letters are significantly different ($p \leq 0.05$).

Sensory evaluation

The chemical stability of meat during storage affects the sensory parameters. In general, sensory parameters are considered the most important factors in using any antibacterial nanoparticles. The effect of nanoparticles on overall acceptability (odor, color, and texture) of minced meat during refrigerated storage at 4 °C is presented in table 3. Sensory properties were satisfactory for all the samples on the initial day of the storage (1st day), however, they decreased during the storage period ($p \leq 0.05$). The results showed that all sensory attributes of control samples were acceptable by the 3rd day of the storage period and spoiled at 6th day; while treated samples were acceptable by the 15th day of storage for texture, color, and overall acceptability attributes, by the 12th day of storage for odor attribute. There was a significant difference ($p \leq 0.05$) for overall acceptability attribute between the treated and control samples on the days 3rd, 6th, 9th, 12th, 15th, and 17th of the storage time. The concentrations of 12 mM ZnO, 12mM TiO2, and the mixture of ZnO + TiO2 (6mm) enhanced shelf lifetime of minced meat and delayed its spoilage until 17th day, while minced meat treated with concentrations of 6mM ZnO and 6mM TiO2 spoiled on 15th day. Similarly, it is reported that the use of TiO2 delay spoilage of meat to 15 days (Alizadeh-Sani et al., 2020). Sensory evaluation of meat products allows researchers to evaluate how consumers perceive associated palatability. The reactions during lipid oxidation can lead to the formation of off-odors and off-flavors, texture, and color changes due to the myoglobin oxidation cause discoloration, which influences consumer's choice and acceptance. Aldehydes are important toxic compounds resulted during lipid oxidation cause organoleptic changes (Banerjee et al., 2017).

Table 3. Effects of different concentrations of ZnO and TiO2 nanoparticles on overall acceptability of minced meat during storage at 4 °C for 17 days.

Groups	1st day	3rd day	6th day	9th day	12th day	15th day	17th day
Control group	8.85 ± 0.08 ^{a}	5.66 ± 0.33 ^{b}	3.66 ± 0.06 ^{b}	3.10 ± 0.09 ^{c}	2.20 ± 0.06 ^{e}	1.50 ± 0.08 ^{e}	1.10 ± 0.10 ^{e}
12 mM ZnO	8.95 ± 0.03 ^{a}	8.88 ± 0.06 ^{a}	7.66 ± 0.33 ^{a}	7.16 ± 0.16 ^{a}	6.50 ± 0.29 ^{a}	5.66 ± 0.33 ^{a}	3.11 ± 0.06 ^{a}
6 mM ZnO + 6 mM TiO2	8.95 ± 0.03 ^{a}	8.71 ± 0.11 ^{a}	7.50 ± 0.29 ^{a}	7.13 ± 0.13 ^{a}	6.16 ± 0.16 ^{a}	5.73 ± 0.39 ^{a}	2.50 ± 0.06 ^{b}
12 mM TiO2	8.95 ± 0.03 ^{a}	8.56 ± 0.29 ^{a}	7.27 ± 0.30 ^{a}	7.13 ± 0.09 ^{b}	6.20 ± 0.15 ^{b}	5.46 ± 0.26 ^{a,b}	2.10 ± 0.06 ^{d}
6 Mm ZnO	9.25 ± 0.28 ^{a}	8.61 ± 0.06 ^{a}	7.10 ± 0.16 ^{a}	6.00 ± 0.48 ^{b}	5.26 ± 0.12 ^{c}	3.00 ± 0.06 ^{b}	2.00 ± 0.12 ^{c}
6 mM TiO2	8.95 ± 0.03 ^{a}	8.62 ± 0.12 ^{a}	7.10 ± 0.13 ^{a}	6.10 ± 0.06 ^{b}	5.23 ± 0.14 ^{c}	2.50 ± 0.06 ^{b}	2.00 ± 0.14 ^{c}

The values are expressed as Mean ± standard error of three experiments. Means within a column and rows followed by different letters are significantly different ($p \leq 0.05$).

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CONCLUSION

It is concluded that nanoparticles have an antimicrobial effect against *E. coli* and their effect is concentration-dependent. It is demonstrated that 12 mM ZnO nanoparticles had a greater antimicrobial effect against *E. coli* than the mixture of ZnO + TiO2 (6 mM), followed by 12 mM TiO2. It is found that these nanoparticles can prevent bacterial growth and enhance the shelf life of minced meat.

DECLARATIONS

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Authors' contributions

Hemmat M. Ibrahim and Rasha Elsabagh designed the plan of study, revised the research article. Mohebat A. Abd El- Aziz, Rasha Elsabagh and Nahla Abo EL-Roos analyzed the data, performed laboratory experiments, and drafted the manuscript. Nahla Abo EL-Roos provided the experimental tools, revised the research article, Badawi Anis helped in the synthesis of nanoparticles and helped in the analysis of TEM pictures.

Competing interests

The authors declare no conflicts of interest.

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