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(Original Article)

Metalonaonoparticles: Friends to Probiotics, Enemies to Pathogenic Bacteria in Dairy Sector

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Abstract

Food spoilage represents a critical issue, only microbial spoilage contributes to 25% loss in the global food industry. Zinc oxide nanoparticles (ZnONPS) and magnesium oxide nanoparticles (MgONPS) serve much attention to their antibacterial activity besides their safety as generally recognized as safe (GRAS) agents that are approved to use in food technology. Concentrations of (25, 50, and 100 µg/ml) from ZnONPS and MgONPS were subjected to six common food pathogens and four probiotic bacteria. Results show well the antibacterial potential for all the pathogenic bacteria, with inhibition zone ranging between 20.200±0.374 to 36.4±0.400 mm for Gram-positive *Staphylococcus aureus* and *Bacillus cereus* and from 11.8 ±0.200 to 40.8±0.374 mm for Gram-negative *E. coli*, *Salmonella sp.*, *Serratia sp.,* and *Pseudomonas sp*. Selected nanoparticles did not affect probiotic bacteria belonging to *Bifidobacterium*.

Here, we introduce green synthesis ZnONPS and MgONPS as preservatives and dietary supplements, their attractive properties of inhibiting foodborne pathogens and maintaining probiotics qualify them to be promising antibacterial agents in dairy industry.

Keywords: *ZnONP, MgONP, Pathogenic bacteria, Bifidobacterium, dairy preservative.*

Introduction

Milk contains several elements; twenty minerals are found to be essential in human nutrition. Essential minerals are classified into two groups (macro-minerals) and trace elements (micro-minerals). The content of elements in milk depends upon many factors such as species, individual animal, feeding method, lactation stage, and health condition of the udder (Cashman, 2011). Zinc is a key trace element; it represents a necessary component of numerous enzymes and metalloproteins. It performs multifunction in the body as DNA repairment, gene expression, cell growth and replication, lipid and protein metabolism, immunological function, and hormonal action (Murakami and Hirano, 2008). The content of zinc differed with milk types, it was (74-145), 242,415, and 0.38 mg/100 g for cow, goat, sheep, and human milk, respectively (Cashman, 2011).

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Nowadays, a great concern has been attuning to the lack of zinc in the human diet, its lack disturbs many functions and causes diseases like loss of appetite, disturbance in smell and taste, inflammation bowel disease, arteriosclerosis, decreased immunity, and hemolytic disease such sickle cell anemia (Prasad, 2013). In addition, magnesium (Mg) plays a vital role in human biology. The content of Mg was (9-16), (10-21), (8-19), and (3-3.4) mg/100 g for cow, goat, sheep, and human milk, respectively (Pietrzak-Fiećko and Kamelska-Sadowska, 2020). Deficiency of Mg is involved in uncontrolled blood pressure (Sontia and Touyz, 2007). Two forms of Mg are investigated as dietary supplementary, the first as magnesium oxide (MgO) and the second as magnesium hydroxide (MgOH) the two forms of Mg are employed as buffering agents in the production of canned vegetables and dairy products. Additionally, they enhance flowability and prevent clumping in dairy breakfast cereal salt and powder concentrates like soft drink mixes (Wetteland *et al.*, 2018). To achieve zinc and magnesium vital roles adults should take from 12 to15 mg (Singh and Garg, 2006) and 420 mg per day (Mirhosseini and Afzali, 2016), respectively.

Food spoilage is considered a great issue, microbial spoilage alone contributes to the loss of about 25% of the food produced globally. Bacteria contaminate several foods and cause food poisoning or food spoilage, this clearly appeared in the dairy industry (Bondi *et al.*, 2014).

Fortunately, metaloxidenanoparticles can display effective antibacterial activity against only one type of bacteria either Gram-negative or positive depending on how nanoparticles and cell wall react. However, green synthesis-assisted ZnONPS and MgONPS show greater antibacterial efficacy against both types of bacteria (Slavin *et al.*, 2017). The antimicrobial action began when metalonanoparicle and bacteria came into contact. ZnONPS cation characteristics allow them to electrostatically connect to the opposite charged bacterial cell which causes accumulation of ZnONPS on the cell wall surface of microbes and results in cellular damage (Slavin *et al*., 2017). Nanoparticles of certain elements have made them considered distinctive and special materials, chemical and physical methods are well-known methods for the synthesis of nanoparticles for preparing zinc nanoparticles. ZnONPS can be synthesized by *Cassia angustifolia* leaf extract (Albrahim *et al*., 2021), *Lb. gasseri* (El-Sayed *et al*., 2021), and *Cucumis melo* (Archana *et al*., 2022). MgONPS can be prepared by using herbal extracts, fruit extract, and mushroom extract (Nejati *et al*., 2022). Nanoparticles can be used in different applications including foodstuff, medication, sunscreen and cosmetics, and antimicrobial and anticancer agents (Jeevanandam *et al.,* 2018).

Zinc oxide (ZnO) and MgO are considered “generally recognized as safe”, therefore, ZnO and MgONPS/MPS have a promising potential for use in many applications including food, medicine, and cosmeceuticals that are linked to antibacterial properties that overcome drug resistance and avoid food preservatives (Elumalai and Velmurugan, 2015).

This work aims to investigate the antimicrobial activities of three concentrations of ZnONPS and MgONPS (25,50, and 100 µg/ml) on six foodborne pathogens strains (*Escherichia coli*, *Salmonella sp*., *Serratia sp*., *Pseudomonas sp*., *Staphylococcus aureus*, and *Bacillus cereus*) besides the effect of fortification of metal-oxide nanoparticles (ZnONPS and MgONPS) on four beneficial bacteria belonging to *Bifidobacterium* to their future application in fermented dairy products.

Materials and Methods

Materials

ZnO and MgO were purchased from Sigma, Aldrich as precursors for ZnONPS and MgONPS preparation.

Bacterial strains

Probiotic bacteria *Bifidobacterium angulatum 2238, Bifidobacterium bifidum LMG 10645, Bifidobacterium breve LMC 017,* and *Bifidobacterium longum ATCC 15707* were provided by Cairo microbiological resources center (MERCIN), Faculty of Agriculture, Ain shams university. Six pathogenic bacteria*: Escherichia coli, Salmonella sp., Serratia sp., Pseudomonas sp., Staphylococcus aureus,* and *Bacillus cereus were* obtained from Microbiology Department, Faculty of Agriculture, Minia University.

Methods

Preparation of ZnONPS

Zinc oxide nanoparticles were obtained through the green synthesis method using green tomato extract as previously synthesized and characterized via the procedure explained by Abdallah *et al.,* (2020). Briefly, two grams of dried green tomatoes were placed in a 250 ml beaker with 200 ml deionized water which was then boiled in a water bath at 60ºC for 4 hrs. Then, filtered the extract twice by filter paper Whatman No. 1 of. ZnONPS were centrifuged at 10,000 g for 20 min. The pellet was washed with distilled water. The nanoparticle powder was obtained by freeze-drying in ALPHA 1-2/LD-Plus vacuum for 8 h.

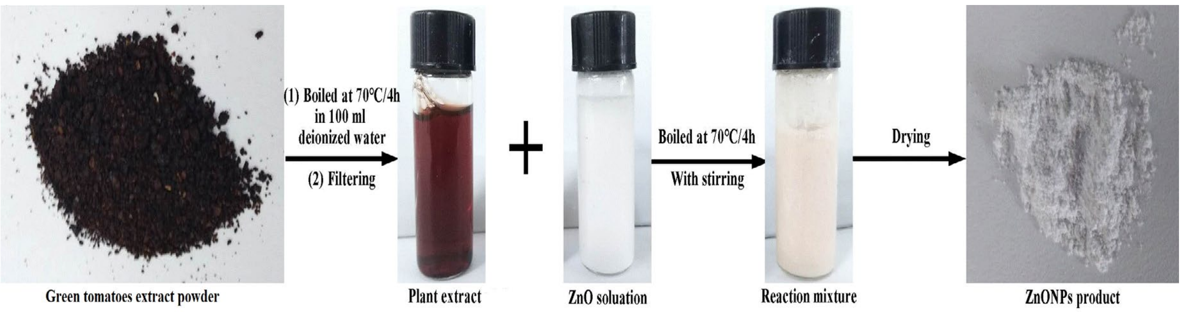

Figure 1. Flowchart diagram of green synthesis of ZnONPS using tomato extract

Preparation of MgONPS

Green synthesis of MgONPS using rosemary extract as previously synthesized and characterized through the biological technique explained by Abdallah *et al.,* (2019) Fig (2). Briefly, Rosemary flowers were purchased from a local market and dried before being pulverized in a home blender. The ground flowers were vacuum-sealed and kept at a temperature of below 10°C in a household freezer. Aliquots of 100 ml of distilled water were used in cooking one gram of ground Rosemary for four hours at 70°C. After the extract have been filtrated via Whatman filter paper No. 1, the yellow-brown extract was gathered for additional testing. One hundred ml of MgO aqueous solution (1.0 mM) was combined with 100 ml of the extract and swirled continuously at 600 rpm at 70°C for four hours by a magnetic stirrer (Magnetic Stirrer, Jiangsu, China). After centrifugation of the mixture at 5000 rpm for 15 minutes to separate it, the precipitate was washed with distilled water before being dried in ALPHA 1-2/LD-Plus vacuum.

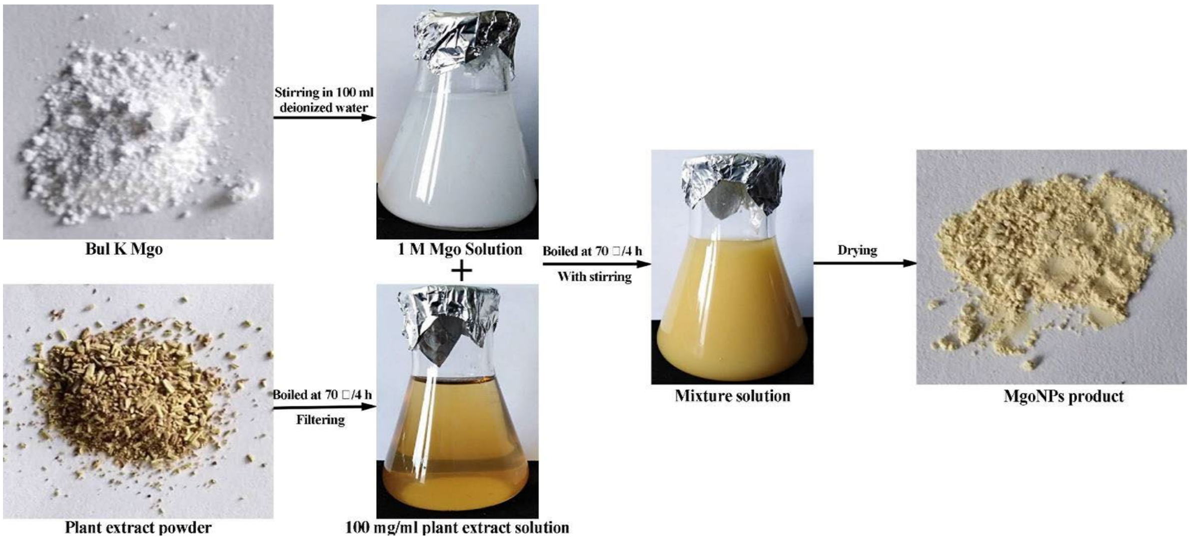

Figure 2. Flowchart diagram of green synthesis of MgONPS using rosemary flowers extract

Determination of antibacterial properties

The biological activity of the prepared ZnONPS and MgONPS was investigated against six Gram-positive bacteria: *Staphylococcus aureus, Bacillus cereus, Bifidobacterium angulatum 2238, Bifidobacterium bifidum LMG10645, Bifidobacterium breve LMC 017,* and *Bifidobacterium longum ATCC 15707* and four Gram-negative bacteria: *Escherichia coli, Salmonella sp., Serratia sp.,* and *Pseudomonas sp*. The agar well diffusion technique method by Lorentz *et al.*, (2006) was followed to examine the antibacterial characteristics of ZnONPS and MgONPS. Using MRS agar for bifidobacteria and nutrient agar for pathogens. The bacteria were let to grow on nutrient agar or MRS agar. The 6 mm well, contains 30 µl of three distinct concentrations: (25,50 and 100 µg/ml). The inoculum was applied to the agar surface, then incubated for 24 h at 37 ̊C. The zone of inhibition that appeared to surround the well was measured in mm.

Statistical analysis

Each evaluation was carried out at least five times, and means and standard errors have been calculated results statically analyzed by two-way ANONA using costat software.

Results and Discussion

Antibacterial characteristics of ZnONPS on food pathogens

The inhibition of ZnONPS on plates was checked against six pathogenic bacteria Fig. 3 and 5. The antimicrobial effect is different as it is strain-dependent. At the concentration of 25 µg/ml, inhibition zones varied between 11.8±0.200 and 32±0.316 mm for the examined strains. Additionally, the inhibitory effect zones are elevated with rising concentration to (50µg/ml), and inhibition zones extended between 14.6±0.244 to 34.8±0.489 mm. Consequently, the highest inhibition zones were recorded at 100 µg/ml were from 19.8±0.538 to 40.8±0.374 mm. *Salmonella sp.* is the most inhibited strain by ZnONPS however, *Pseudomonas sp.* was the most resistant strain at 100 µg/ml. Joe *et al.*, (2017) explained that antimicrobial activity initially emerges by metalonanoparicle and bacteria interaction. Thus, qualify ZnONPS to attach to the bacterial cell surface that is charged negatively via electrostatic interaction leading to collection of ZnONPS on the bacterial surface and creating cell death.

In general, metaloxidenanoparticles will show clear antibacterial features against either Gram-negative or positive according to the interaction of nanoparticles with the cell wall (Slavin *et al.*, 2017). But *Cucumis melo* assisted to show an excellent antibacterial effect towards (*S. aureus* and *E. coli*) which emphasize that ZnONPS are able to penetrate both kinds of the bacterial cell membrane. *E. coli* has dense impermeable cell membrane lipopolysaccharide that could not be damaged easily by chemical compounds. However, the generated ZnONPS damage can this membrane and suppress the growth through Zn2+ releasing or due to ROS formation that destroyed the cell wall. Moreover, the nanoparticle size impacts a crucial part of bacterial membrane inhibition (Archana *et al.*, 2022).

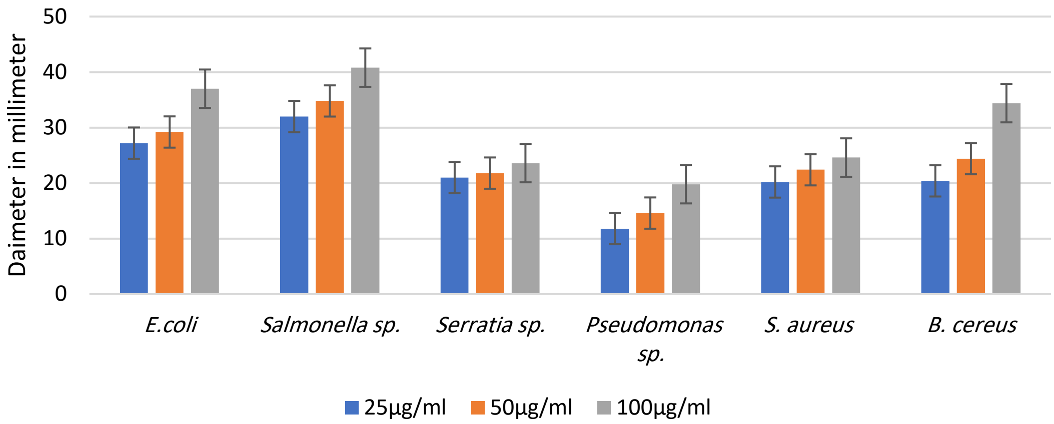

Figure 3. Antagonism in millimeters diameter zone of ZnONPS against Gram-negative and Gram-positive pathogenic bacteria

Green synthesis ZnONPS exhibit antimicrobial action against numerous *Candida* species as well as Gram-positive and Gram-negative bacteria (Elumalai and Velmurugan, 2015). The observed inhibition zone for *E. coli* was 6.70 ± 0.46 mm at 100 µg/ml, which was higher than those obtained for *S. aureus* 6.20 ± 0.43 (Archana *et al*., 2022), these findings in accordance with the present study 37±0.316 and 24.6±0.244, respectively. Archana *et al*., (2022) examined the antibacterial behavior of ZnONPS versus *S. aureus, E. coli,* and *Pseudomonas* their results are lower than that conducted in this study, variations may be due to preparation method and particle size.

El-Sayed *et al*., (2021) studied the antimicrobial effect of ZnONPS on five bacterial strains *Salmonella typhimurium*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Yersinia enterocolitica*, and two fungi *Aspergillus niger* and *Candida albicans*, at 20 mg/ml inhibition zones were from 20 to 34 and at 30 mg/ml was from 20 to 37 mm. Diameters are similar to that recorded in the current study despite the lower concentrations that may be due to the particle size and the method of preparation. The nanoparticles' size within these ranges can freely penetrate the bacterial cell membrane causing damage (Raghupathi *et al*., 2011). Metaloxidenanoparticles encourage the release of ROS that suppress some enzymes in the respiratory chain of the microbe. Accumulation of hydroxyl radical, singlet oxygen, superoxide anions, and hydrogen peroxide can destroy proteins and DNA (Tang and Zheng, 2018).

Antibacterial characteristics of MgONPS on food pathogens

The antimicrobial action of MgONPS is presented in Fig. 4 and 6. The inhibition zone diameter was in the range of 16.2±0.374 to 30±0.316 mm for all tested strains at 25 µg/ml. Moreover, a larger concentration (50µg/ml), resulted in a higher inhibition zone in diameter of 24.2 ±0.374 to 32.2±0. 0.200 mm. Consequently, the highest inhibition zones were noticed at 100 µg/ml that was from 24.8±0.200 to 36.4±0.400 mm. In the consideration of MgONPS, *Serratia sp.* is the most inhibited strain while *Pseudomonas sp.* was the most resistant strain at 100 µg/ml.

Li *et al*., (2022) approved the antibacterial effect of MgONPS on *E. coli* and *S.aureus*. Antibacterial action of synthesized MgO nanoflakes against *Staphylococcus aureus*, as a potential antibacterial agent against *S. aureus* at a concentration below 250 µg/ml, and the proposed mechanism was explored by surface morphological analysis using scanning electron microscopy (Das *et al*., 2018). The obtained results are in agreement with these findings, MgONPS could inhibit *S. aureus* at concentrations from 25 to 100 µg/ml. The antibacterial activity is enhanced by decreasing particle sizes and increasing the surface. In addition, Huang *et al*., (2005) revealed that the MgO inhibition against *Bacillus subtilis* was improved upon MgO particle size was decreased. Mirhosseini and Afzali, (2016) investigated the antibacterial action of MgONPS alone or in combination with nisin or ZnONPS against *Salmonella Stanley* and *E. coli O157:H7*. The results indicated that MgONPS has superior bactericidal activity. The addition of nisin to MgONPS enhanced antibacterial performance. However, the combination of

ZnONPS with MgONPS altered the antibacterial action of MgO against both Gram-negative studied pathogens. Generation of ROS and alkaline effect have been proposed to explain the antibacterial mechanism of MgO nanoparticles (Jin and He, 2011).

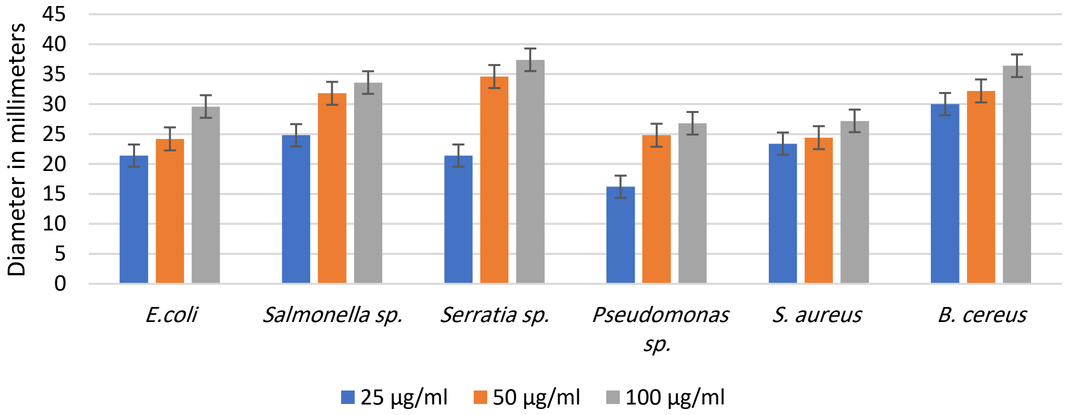

Figure 4. Antagonism in millimeters diameter zone of MgONPS against Gram-negative and Gram-positive pathogenic bacteria

El-Mekkawi *et al.*, (2018) compared the antibacterial effect of three metal oxides on Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) using the agar diffusion method. Overall, both copper oxide nanoparticles (CuONPS) and ZnONPS revealed better antibacterial performance than MgONPS, these results are on the contrary side of the current study MgONPS exceeded ZnONPS, and results statically differ ($p \leq 0.05$).

Effect of ZnONPS and MgONPS on bifidobacteria

Interestingly, ZnONPS and MgONPS nanoparticles showed a marvelous feature that they did not have any antagonistic effect on *Bifidobacterium angulatum 2238, Bifidobacterium bifidum LMG10645, Bifidobacterium breve LMC 017* and *Bifidobacterium longum ATCC 15707* up to 100 µg/ml. Fig (5 and 6). Zhou *et al.*, (2021) conducted a study to know how ZnONPS affect the gut microbiome of ADHD children, results revealed that bifidobacterial count was still constant in the presence or absence of ZnONPS at a level of 20 µg/ml. In contrast, gut microflora composition was changed, thus meaning that bifidobacteria are resistant to ZnONPS. The use of microorganisms in the synthesis of ZnONPS is common namely an eco-friendly method. Several lactic acid bacteria (LAB) posse mechanisms to survive in the presence of Zn2+ not only abolish their toxicity but also, produce ZnONPS thus, they can be used as nanofactory. LAB are able to interact with Zn2+ either by biosorption or bioaccumulation mechanisms. *Lactobacillus Plantarum* strain TA4, a probiotic strain resists Zn2+ up to 500 mM (32700 µg/ml) and produces ZnO NPS (Mohd Yusof *et al.*, 2020). These findings explain why bifidobacterial did not inhibit by ZnONPS and MgONPS up to 100 µg/ml.

Incorporation of zinc oxide nanoparticles in packaging will be allowed to prevent the microorganisms' development and food degradation in the future (Gudkov *et al.*, 2021). Researchers recommended iron and zinc in the formative of nanostructured components for nutritional purposes as enhanced bioavailability and reduced changes in color in final products (Hilty *et al.*, 2009). ZnONPS and MgONPS prepared by green synthesis pretend to be promising antibacterial agents. Addition of ZnONPS or MgONPS in food, especially fermented dairy products can exert several roles: fortification with the mineral, inhibition of pathogenic bacteria, and maintaining probiotics. Moreover, utilization of ZnONPS and MgONPS in packaging material could prevent pathogenic bacterial growth and extend the shelf life of the products.

Figure 5. Antibacterial action of zinc oxide nanoparticles against pathogenic and probiotic bacteria.

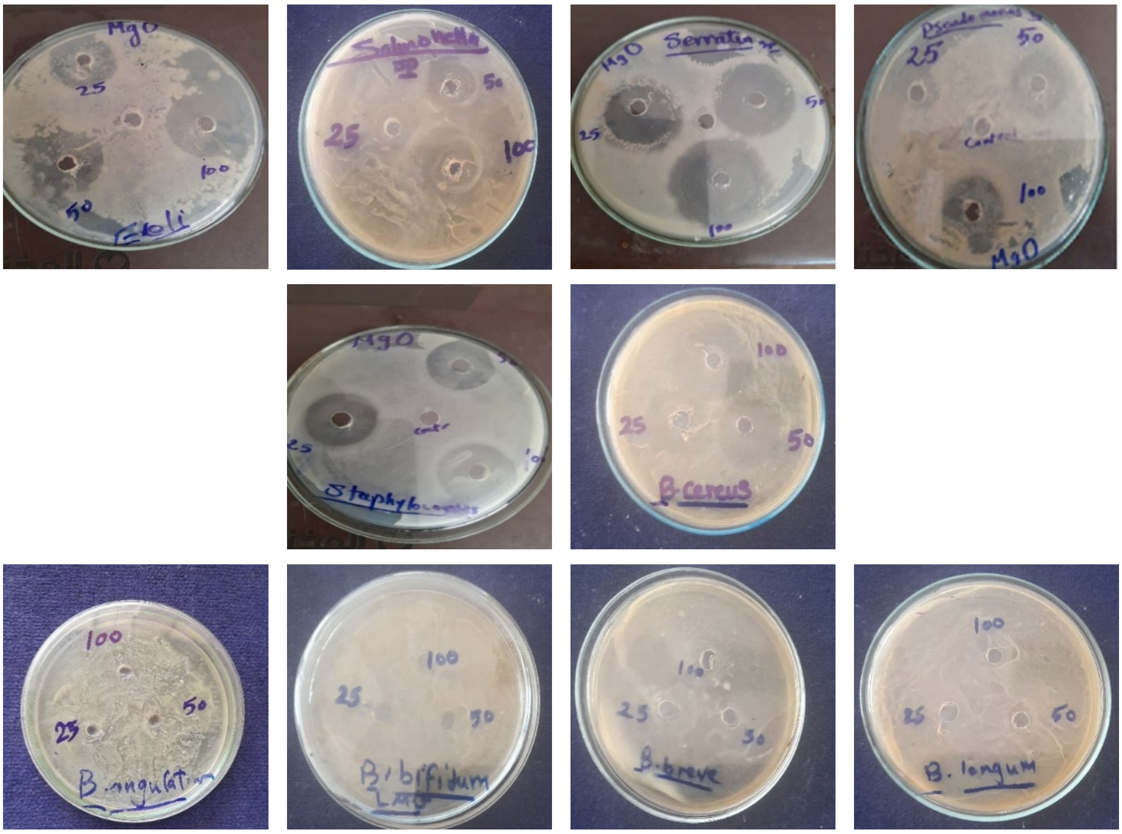

Figure 6. Antibacterial action of magnesium oxide nanoparticles against pathogenic and probiotic bacteria.

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الجزيئات المعدنية النانومترية: أصدقاء لبكتريا البروبيوتيك، أعداء للبكتريا المسببة للأمراض في مجال الألبان

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[2] قسم امراض النبات، كلية الزراعة، جامعه المنيا، المنيا، مصر.

الملخص

يمثل فساد الغذاء مشكلة حرجة، فالفساد الميكروبي منفردا يساهم في خسارة 25٪ من اجمالي الغذاء المنتج عالميا. تجذب الجسيمات النانومتريه لاكسيد الزنك (ZnONPS) واكسيد الماغنسيوم (MgONPS) اهتماما كبيرا بنشاطها المضاد للبكتريا الي جانب مأمونيتها كمواد معترف بها انها آمنه (GRAS) ومصرح باستخدامها في التصنيع الغذائي . اختبرت تركيزات (25،50 و 100 ميكروجرام / مل) من الجسيمات النانومتريه لاكسيد الزنك ZnONPS واكسيد الماغنسيوم MgONPS لستة أنواع من البكتريا المسببة للأمراض المنتقله عن طريق الغذاء الشائعة وأربعة سلالات من بكتيريا البروبيوتيك. اظهرت النتائج فاعليه المواد المستخدمة كمضادات لجميع البكتيريا المسببة للأمراض، مع منطقة تثبيط تتراوح بين 20.200 ± 0.374 إلى 36.4 ± 0.400 مم *Staphylococcus aureus* و *Bacillus cereus* ، ومن 11.8 ± 0.200 إلى 40.8 ± 0.374 مم للبكتريا سالبة الجرام *E. coli* و *Salmonella sp.* و *Serratia sp.* و *Pseudomonas sp.* بينما لم تؤثر الجسيمات النانومترية المختبرة على بكتيريا البروبيوتيك التي تنتمي إلى جنس *Bifidobacterium*.

الجسيمات النانومتريه لاكسيد الزنك ZnONPS واكسيد الماغنسيوم MgONPS المحضرة بالتخليق الأخضر يمكن استخدامها كمواد حافظة ومكملات غذائية، بجانب خصائصهما الجيدة في تثبيط مسببات الأمراض التي تنتقل عن طريق الأغذية والحفاظ على بكتريا البروبيوتيك مما يؤهل هذه المواد لتكون عوامل واعدة كمضادات للبكتريا في الألبان.