



The Effectiveness of Bacteriocin Composites with Nanoparticles against Bacterial Food Contaminants *in vitro*

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A B S T R A C T

The scientific community is currently witnessing a paradigm shift toward integrating nanotechnology with biological components to address the escalating challenges of microbial resistance. Therefore, this study intended to prepare different combinations of bacteriocin-nanocomposites with examining their antagonistic activity toward some foodborne bacteria. In this study, bacteriocin production from *Lactobacillus acidophilus* was optimized across different culture media, temperatures, and pH levels, followed by partial purification via gel filtration chromatography. Meanwhile, magnesium oxide (MgO) and zinc oxide (ZnO) nanoparticles were created via green synthesis using the filtrate of *Escherichia coli*. Then, the nanoparticles were characterized by X-ray diffraction analysis, atomic force microscopy, ultraviolet-visible spectroscopy, fourier transform infrared spectroscopy, and field emission scanning electron microscopy to verify their structural properties. Eventually, the antibacterial activity at specific concentrations of bacteriocins, nanoparticles, and their combinations was tested *in vitro* by the agar well diffusion approach targeting some foodborne pathogens, including *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus* spp. The findings showed that the optimal settings for the bacteriocin production were achieved in the De Man-Rogosa-Sharpe (MRS) broth at pH 6.0 and temperature 37°C. Characterization of the ZnO and MgO nanoparticles revealed spherical shapes and solitary aggregations with average sizes of 62.08 and 46.43 nm, respectively. While MgO and its composite were ineffective against the tested microorganisms, the combined application of bacteriocins and ZnO nanoparticles were superior in inhibiting the bacteria causing wider inhibition zones (27, 29, 23, and 21 mm) toward *S. aureus*, *Bacillus* spp., *E. coli*, and *P. aeruginosa*, respectively, than their use individually. It can be concluded that integration of ZnO nanoparticles with the optimized bacteriocins significantly enhances their antibacterial potential against foodborne pathogens. These *in vitro* findings require further safety examination *in vivo* before being recommended as a bright and sustainable bio-preservative system for the food industry.

Keywords: bacteriocin, ZnO nanoparticles, MgO nanoparticles, green method, antibacterial activity, foodborne pathogens

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INTRODUCTION

Bacteriocins are a heterogeneous group of bioactive peptides secreted extracellularly by bacteria, and

they vary in the molecular weight, ranging from small peptides in class I and II (< 10 kDa) to larger heat-labile proteins in class III (> 30 kDa) (1). These compounds are considered safe for human consumption as they are easily

degraded by gastrointestinal proteases (2). Gram-positive bacteriocins are largely made by lactic acid bacteria (LAB), which are generally recognized as safe (GRAS) by the US FDA without adverse effects on food (3). The production of these bioactive peptides is primarily triggered by quorum sensing mechanisms and environmental stress as a response to microbial competition (4). Furthermore, the efficiency of this production process is strictly determined by optimal pH levels, incubation temperature, and specific media composition that support the metabolic pathways during the late log growth phase (5). Among the LAB, *Lactobacillus*, in particular *Lactobacillus acidophilus*, is highly valued in the food industry for its role not only as a probiotic but also as a powerful source of bacteriocins (6). The bacteriocins' capability to constrain or destroy relevant pathogens, including multi-drug resistant (MDR) bacteria in vitro has been well acknowledged. Applying crude, partly purified, and purified bacteriocin combinations, has been found to significantly reduce food spoilage by inhibiting *Bacillus cereus* and *Staphylococcus aureus*, the two food-borne spoilage-causing pathogens (7). Despite bacteriocins benefits as surrogate therapies over the current strategies, they still have a number of drawbacks that need to be tackled. These limitations involve the expensive isolation and purification, easy enzymatic degradation, decreased stability and solubility, a limited range of activity, as well as the possibility of emerging bacterial resistance against them (3,8). In addition, it has been demonstrated that the use of bacteriocins alone in food is unlikely to guarantee complete safety, particularly when it comes to Gram-negative bacteria (2).

To prevent the issues associated with the direct addition of bacteriocins to food, nanotechnology has been widely used as an innovative technology with enormous potential in various facets of the food industry, e.g., functional food development, food safety, food processing, and food packaging (9). Among nanoparticles, magnesium oxide (MgO) nanoparticles have been demonstrated to provide continuous antimicrobial protection with maintaining a sterile environment around the food (10,11). Additionally, zinc oxide (ZnO) nanoparticles have presented potent antimicrobial properties (12). However, the potential toxicity of these nanoparticles is a key limitation that must be addressed. This toxicity primarily arises from their small size and high surface reactivity, which can lead to the generation of reactive oxygen species (ROS) and induce oxidative stress within cells, potentially damaging proteins, lipids, and DNA (13). Furthermore, the ability of MgO and ZnO nanoparticles to penetrate cellular membranes may interfere with normal metabolic pathways, making their safety profile a critical factor in their application (14,15). Despite the extensive manufacturing of nanoparticles using the physical and chemical methods, employing these nanoparticles for medicinal purposes, especially in clinical situations, has been limited due to the existence of hazardous chemicals, e.g., toxic reducing agents like sodium borohydride and hydrazine, or volatile organic solvents used in traditional synthesis. These substances pose significant risks to human health and the environment,

unlike the eco-friendly "green" bacterial methods. Therefore, developing nontoxic, environmentally friendly, and reliable production methods is essential. One approach is cultivating bacteria as "nanofactories" to produce nanoparticles (16). Because it requires little energy and has less unfavorable influences compared to the chemical techniques, this "green" method is valuable for the society and the environment (17). Furthermore, nanomaterials have become widely used in conjugation with bacteriocins in order to strengthen their impacts or lessen issues associated with their use independently in biomedical implementations (2). Although the bacteriocin-nanoparticle conjugate science is still in its infancy, it has enormous possibility to transform the food industry (2). From what is illustrated above, further research is needed to optimize bacteriocin-nanoconjugate compositions. Thus, this study intended to prepare and examine the activity of different combinations of bacteriocin-nanocomposites in preventing some Gram-positive and Gram-negative bacteria contaminating food, e.g., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bacillus* spp.

MATERIALS AND METHODS

Ethical Approval

This investigation was exempted from the ethical approval certificate because it used bacteria already isolated by another study (18) from minced lamb and soft cheese.

Food-Borne Bacteria

Food-borne microorganisms, including *S. aureus*, *E. coli*, *P. aeruginosa*, and *Bacillus* spp. contaminating local minced lamb and soft cheese were obtained from and identified in the study of Al-Sahaf et al. (18).

Lactobacillus acidophilus and its Molecular Identification

Live active *L. acidophilus* was obtained from the commercial product named 'Natural Probiotic, Pure Acidophilus Yogurt Starter' (NPSelection, EU). This bacterium was cultivated into the De Man-Rogosa-Sharpe (MRS) broth (HiMedia, India) and further analyzed by the PCR assay using primers specific to the MFS-transporter gene (19). The hypothetical PCR products amplified by these primers were also tested for their size using the *in silico* PCR amplification software (<http://insilico.ehu.eus/>) (20).

Before DNA extraction, cells of *L. acidophilus* grown on the MRS agar were harvested with sterile phosphate buffered saline and spun for 1 min at 13,000 rpm to collect cell pellets. Using 'Easy Pure® Genomic DNA Kit' (Transgene®, China), the genomic DNA was extracted from the cell pellets as per the protocol of the supplying company. Components of the PCR mixture involved 12.5 µL (2×) of One Taq MasterMix (NEB, England), 3 µL of the DNA sample, 1 µL (10 pmol/µL) of the forward primer (F) (5'-TCATGTTGGGATGCAATGAG-3') and 1 µL (10 pmol/µL) of the reverse primer (R) (5'-TTTCAAACTTGTCTGCTG-3')

manufactured by MacroGen (Korea), as well as 7.5 μ L of nuclease-free water (NEB®, England). The entire 25 μ L of the reaction components were mixed by pipetting followed by short spinning before running the preset PCR program. The PCR program was set using Thermal Cycler (Eppendorf, Germany) to magnify the target gene. The PCR cycling protocol was set at 94°C for 5 min and one cycle (the initial denaturation step). Then, three steps repeated for 40 cycles were run at 94°C for 20 sec (denaturation), 58°C for 30 sec (annealing), and 72°C for 1.5 min (elongation). Afterward, a step was conducted for 7 min at 72°C (final elongation). Following the PCR technique, the products were further analyzed by the D5000 ScreenTape assay on an Agilent TapeStation system (Agilent, Germany). All the reagents and the system were equilibrated to room temperature for 30 min before use. For sample preparation, 1 mL of the DNA was added to 10 mL of D5000 Sample Buffer (Waldbronn, Germany). Similarly, the ladder was prepared by mixing 1 mL of D5000 Ladder (Waldbronn, Germany) with 10 mL of the Sample Buffer. The prepared combinations were vortexed and spun at 2000 rpm for 1 min before automated electrophoresis and analysis by the TapeStation Controller software. This step was conducted at the Department of Molecular Pathology, University of Southampton General Hospital, Southampton, UK. Then, the 16S rRNA gene of the above bacterium was also amplified using primers targeting V3-V4 variable regions (NC_000913.3): 341F-CCTACGGGNGGCWGCAG and 805R-GACTACHVGGGTATCTAATCC (21). For the PCR assay, 15 μ L of the master mix (KAPA HiFi Hot Start Ready Mix PCR Kit; KK2602, Roche-Germany) was used. The reaction also contained 5 μ L of the bacterial genomic DNA (10 ng/ μ L), 0.5 μ L of a solution containing bovine serum albumin (20 mg/mL) in 50 mM Tris-HCl, 0.1 M NaCl, 0.25 mM EDTA, 1 mM 2-mercaptoethanol, and 50% glycerol (v/v) at pH adjusted to ~ 7.6 to supports the polymerase enzyme stability. Additionally, 4.5 μ L distilled water and 2 μ L of the F and R primers were also added. The cycling conditions, using the same Thermal Cycler, started with the denaturation step at 98°C for 2 min. This was followed by denaturation at 98°C for 20 sec, the annealing step at 54°C for 20 sec, and the elongation process at 72°C for 15 sec, for 28 cycles each. The Final elongation was set up at 72°C for 2 min, and the amplicons were stored at -20°C till use. Then, the 16S rRNA gene sequences were generated using the Sanger method (MacroGen corporation, Korea), and this was followed by bioinformatics sequence analysis and GenBank registration.

Bacteriocin Production and Purification

Production of bacteriocin was performed as per the procedure of Al-Jumaily et al. (22). In brief, a crude bacteriocin was produced by culturing *L. acidophilus* in the MRS broth (HiMedia, India), whose pH was amended to 5.5 by adding 1 N HCl, and the culture incubation lasted for 48 h at 37°C. Then, the bacterial cells were gathered by spinning at 6000 rpm for 10 min, and the cell-free supernatant was named crude bacteriocin, which was heated at 80°C using a water bath (Grant, England) for 10

min. Later, after cooling the supernatant, it was completely mixed 1:1 with n-butanol, and hold for 24 h at the refrigerator temperature. Then, the mixture was spun for 10 min at 4000 rpm to attain organic and aqueous phases. A rotary evaporator at 80°C was used to evaporate the organic phase, and 20 mM sodium citrate buffer (BDH, England) was employed to resuspend the sediment. Then, the protein concentration of the crude bacteriocin was established by the Bradford method (23), followed by estimating its antibacterial activity.

The re-suspended solution was partly refined by gel filtration chromatography using a column measuring 2 × 59 cm (Sephacryl S-1000 superfine; Cytiva, Sweden). Before packing, the dry Sephacryl medium was fully swollen (hydrated) by adding an adequate volume of sodium citrate buffer followed by using a vacuum degasser (Senon, China) to remove all trapped air from the swollen gel and the buffer. This was a critical step to prevent air bubbles from forming in the column bed, as this would cause uneven flow. Then, to avoid bubble formation, the gel was poured gradually by a glass rod into the column. The column was equilibrated with 25 mM sodium citrate buffer, and the bacteriocin sample was poured onto the column walls. After that, 5 mL from each fraction was examined at an absorbance of 280 nm using a spectrophotometer-UV (Dabble Beam, Japan). The active fraction was then collected in a tube, the antibacterial effect was tested by the agar well diffusion method, and the protein concentration was determined as stated above. To confirm the proteinaceous nature of the inhibitory agent and to establish its identity as a true bacteriocin, a targeted proteolytic sensitivity assay was performed on the partially purified fraction obtained from the gel filtration. This partially purified bacteriocin was treated with proteinase K enzyme (Thermo Fisher, USA) at a final concentration of 1 mg/mL. The mixture was incubated at 37°C for 1 h to allow for potential enzymatic cleavage of peptide bonds. After incubation, the antibacterial activity was determined using the agar well diffusion method against *Bacillus* spp., isolated in this study, as an indicator strain. A complete loss of the activity following proteinase K treatment was taken as definitive evidence of the proteinaceous nature of the agent.

Optimization of Bacteriocin Production

For determining the influence of the incubation temperature, pH, and different culture media on the bacteriocin production, various conditions were chosen and tested. The temperature degrees tested were 35°C, 37°C, 39°C and 41°C; and the pH values were 2, 4, 6, and 10. While the culture media involved nutrient broth, tryptic soya broth (TSB), MRS broth, and Brain Heart Infusion (BHI) broth (all from HiMedia, India). An isolate of *E. coli*, already obtained from contaminated foods of this study, was used as an indicator to select the optimal conditions that made the bacteriocin produced having the highest antibacterial activity. Concisely, the agar well diffusion assay was done according to Barrow and Feltham (24), in which an *E. coli* isolate was cultivated aerobically overnight

at 37°C in the nutrient broth. The bacterial growth was then diluted by sterile normal saline to be approximately equivalent to 0.5 McFarland tube. Afterward, 0.1 mL of the bacterial suspension was distributed evenly on the nutrient agar (HiMedia, India) plates, which were left to dry within 10 min, and 6 mm equidistant wells were made. The wells were then filled with 50 µL of the crude bacteriocin. The inhibition threshold was defined as the minimum concentration that produced a visible zone of inhibition (> 6 mm). This threshold was used as a quantitative marker during the optimization of pH, temperature, and media; the conditions yielding the largest diameters across the dilution series were identified as optimal. Any result equal to the well size (6 mm) was considered below the effective threshold.

Synthesis of ZnO and MgO Nanoparticles

The MgO and ZnO nanoparticles were synthesized at the Nanotechnology Laboratory, Department of Biotechnology, College of Science, University of Baghdad. Using a green synthesis approach, these nanoparticles were prepared from inorganic precursors: zinc chloride and magnesium chloride. To obtain the biological reducing agent, *E. coli*, isolated in this study, was cultured in a nutrient broth and incubated at 37°C for 24 h. The culture was then centrifuged at 6000 rpm for 10 min to obtain the bacterial supernatant. The subsequent synthesis process was adapted from El-Shanshoury et al. (25) with some modifications, which involved adding 1 g of each metal salt separately to 10 mL of the bacterial filtrate. The mixture was placed in a shaking incubator (Jissy, Korea) overnight at room temperature to facilitate precipitation. The resulting precipitate was then collected by centrifugation at 6000 rpm for 10 min and washed twice with 95% ethanol to remove any residual chlorides or filtrate traces. Finally, the purified precipitate was transferred to a glass dish and dried in an oven at 40°C.

Characterization of MgO and ZnO Nanoparticles

Several analytical techniques were used to analyze the nature, chemistry, and morphology of the manufactured nanoparticles, such as fourier transform infrared spectroscopy (FT-IR, SHIMADZU, Japan), which used a resolution of 4 cm⁻¹ and a wave number range 4000-400 cm⁻¹ to identify the functional groups composition on the surface of the synthesized nanoparticles. Using this techniques, the absorption of IR by the dried material was estimated and plotted against the wavelength. Field emission scanning electron microscopy (SEM, S-4160, Japan) was used to determine the crystallinity, structural surface distribution, size, dispersion, and morphology of the nanoparticles. X-ray diffraction (XRD, SHIMADZU, Japan) was also accomplished to assess the size and the crystal structure of the nanoparticles using UV-Vis spectrophotometer (at the radiation line Cu Kα and a wavelength of 1.54 Å in 2θ). Ultraviolet-visible absorption spectroscopy (UV-Vis, SHIMADZU, Japan) using spectrophotometer was applied to measure the wavelength of the reaction mixture at a resolution of 1 nm in 2 mL quartz cuvette with 1cm path length. The samples were

tested at a scan speed of 475 nm/min and scanning range of 200-800 nm. Finally, Atomic force microscope (AFM, Phywe, Switzerland) was utilized to visualize the surface morphology and diameter of the nanoparticles with the aid of scanning prob microscopy (NT-MTD, Russia).

Evaluation of the Antibacterial Activity

The antibacterial effect of bacteriocin, ZnO nanoparticles, MgO nanoparticles, bacteriocin-ZnO nanocomposite, as well as bacteriocin-MgO nanocomposite was tested in vitro against the food-borne bacteria. Pure cultures of the bacterial isolates (*E. coli*, *P. aeruginosa*, *S. aureus*, and *Bacillus* spp.) were used. The agar well diffusion assay used to test these bacteria was similar to that applied to the indicator *E. coli* mentioned above in bacteriocin optimization. Except that these isolates were tested against numerous concentrations (100, 50, 25, 12.5, 6.25, 3.125, and 1.56 mg/mL) of bacteriocin, nanoparticles, or bacteriocin and nanoparticles composite. In addition, two wells of the same size were made in another plate and filled with the same amount of deionized water or the MRS broth as a negative control. Afterward, the plates were incubated aerobically for 24 h at 37°C, and the diameter of the inhibition zone surrounding each well was estimated using a ruler to assess the efficiency of each concentration. Experiments of the bacterial inhibitory activity were conducted in triplicate.

Statistical Analysis

All results of the antibacterial activity were reported as a mean ± standard deviation (SD). Two-way analysis of variance (ANOVA) was used to assess the significant differences between the compounds under test and their different concentrations. Duncan's Multiple Range Test (DMRT) was employed to compare means at a confidence level of 95%. The threshold for statistical significance was set as $P < 0.05$. Additionally, SAS software (Version 9.6th; SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

RESULTS

Bacteriocin Production and Partial Purification

Prior to bacteriocin production, *L. acidophilus* was successfully grown and identified to the molecular level using the PCR assay followed by the D5000 ScreenTape assay and the 16S rRNA gene sequencing. Regarding the gel image (Tape Station Electropherogram) shown in **Figure 1A**, it reveals sharp bands of ~649 bp. In comparison, the other two closest sizes (668 bp and 659 bp), appeared in **Figure 1B**, they just represent smears located above the accurate bands. The identity of the bacterium was further verified by its 16S rRNA gene sequence, which was successfully registered within the GenBank under the accession number PZ389740. Then, the bacteriocin was effectively purified partially from *L. acidophilus* via a four-step procedure. The findings revealed that the specific activity and the yield % of the crude bacteriocin and that of the bacterial filtrate following its heating to 80°C was the

same, i.e. 80 Au/mL, with a yield % of 100%. Pertaining to the protein concentration, the crude and the heat treated filtrate reported 10.6 and 9.6 mg/mL, respectively. Meanwhile, the specific activity and the protein concentration of the crude bacteriocin increased after being treated with n-butanol, reaching 320 Au/mL and 67.6 mg/mL, respectively, and the purification fold increased by 0.6 with a yield by 40%. In addition, the gel filtration results between the fractions 17-28 revealed that the specific activity reduced to 160 Au/mL and the protein concentration dropped to 11.5 mg/mL. Afterwards, the proteinaceous nature of the inhibitory agent produced by *L.*

acidophilus was successfully confirmed by the targeted protease sensitivity assay (**Figure 2**). The partially purified bacteriocin (obtained after gel filtration) exhibited a strong antimicrobial activity against the indicator *Bacillus* spp., with a mean inhibition zone of 32 mm. Upon treatment with the proteinase K, the antimicrobial activity was completely abolished, resulting in no inhibition zone. Furthermore, the negative control (proteinase K alone) showed no inhibitory effect against the indicator isolate. These findings rigorously establish the identity of the inhibitory agent as a true bacteriocin, as its activity was directly dependent on its intact peptide structure.

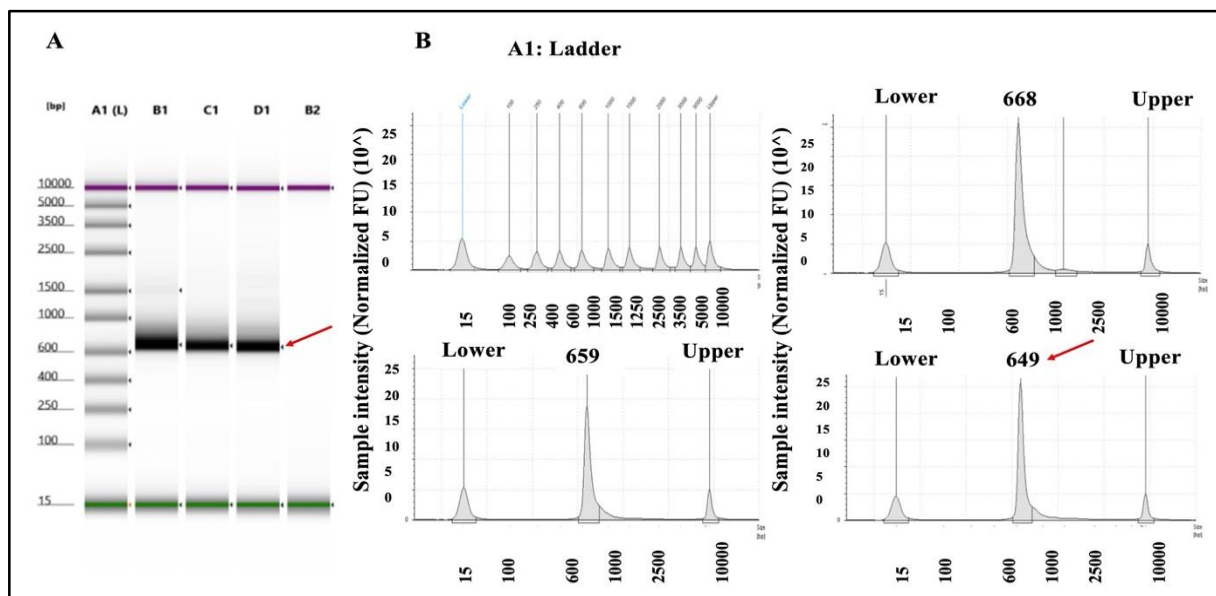


Figure 1. Assessment of the concentration and quality of the input DNA product using the TapeStation system after the PCR amplification process. (A) The gel image shows the concentration and size of the target DNA in three sample replicates. (B) Electropherogram of the DNA product represents the quantification of the isolated DNA. The red arrow indicates the correct PCR product

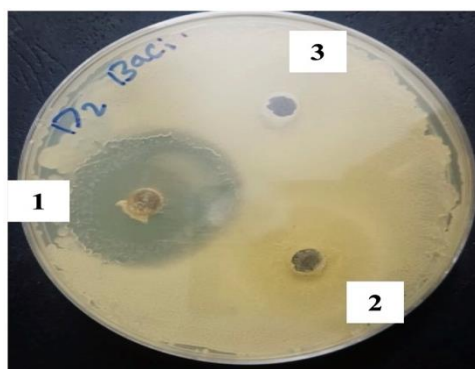


Figure 2. Verification of the proteinaceous nature of bacteriocin either treated or not with proteinase K through testing its inhibitory activity against *Bacillus* spp. 1: well filled with partially purified bacteriocin without enzyme treatment. 2: well filled with partially purified bacteriocin treated with the enzyme. 3: well contained the enzyme only as a negative control.

Optimization of Bacteriocin Production

The indicator bacterium, *E. coli*, successfully helped to choose the various conditions under which the bacteriocin

synthesized from *L. acidophilus* showed the robust antibacterial activity. For instance, the use of 37°C as an incubation temperature for the crude bacteriocin production exerted the best results, where a considerable

zone of inhibition (~ 17 mm) occurred against *E. coli*, compared to the bacteriocin produced under the different incubation temperatures. An inhibition zone of ~ 7 mm noticed when a temperature of 35°C and 39°C were used, and no zone at all upon the use of 41°C. Concerning the best medium that caused the maximum production of bacteriocin with the highest bacterial inhibitory effect, there was not a major difference between the media used. The bacteriocin synthesized by *L. acidophilus* grown into the MRS, nutrient broth or BHI caused *E. coli* inhibition zones of approximately 17, 16, and 15 mm, respectively. On the other hand, the bacteriocin produced from bacteria cultured into TSB was not very effective since it showed the least bacterial inhibition (9 mm). Considering the influence of the pH on the bacteriocin synthesis in the MRS broth, the optimum pH was found to be 6, where inhibition zones of ~ 17 mm occurred. While pH 2 resulted in low zones of

growth inhibition, both pH 4 and pH 10 were not suitable for the bacteriocin production.

Production and Characterization of MgO and ZnO Nanoparticles

UV-Vis Spectrophotometer

The optical features of the biosynthesized MgO and ZnO nanoparticles are shown in **Figure 3 (part1)**. The accomplished UV-Vis crest demonstrated a coordinated electrons recombination between the valence and conduction groups in both MgO and ZnO nanoparticles at an absorbance range of 280 nm and 270 nm, respectively. The present study suggests that the nanoparticles' purity and crystalline nature are reflected in the sharpness of the absorbance peak within this range, indicating a consistent particle size distribution.

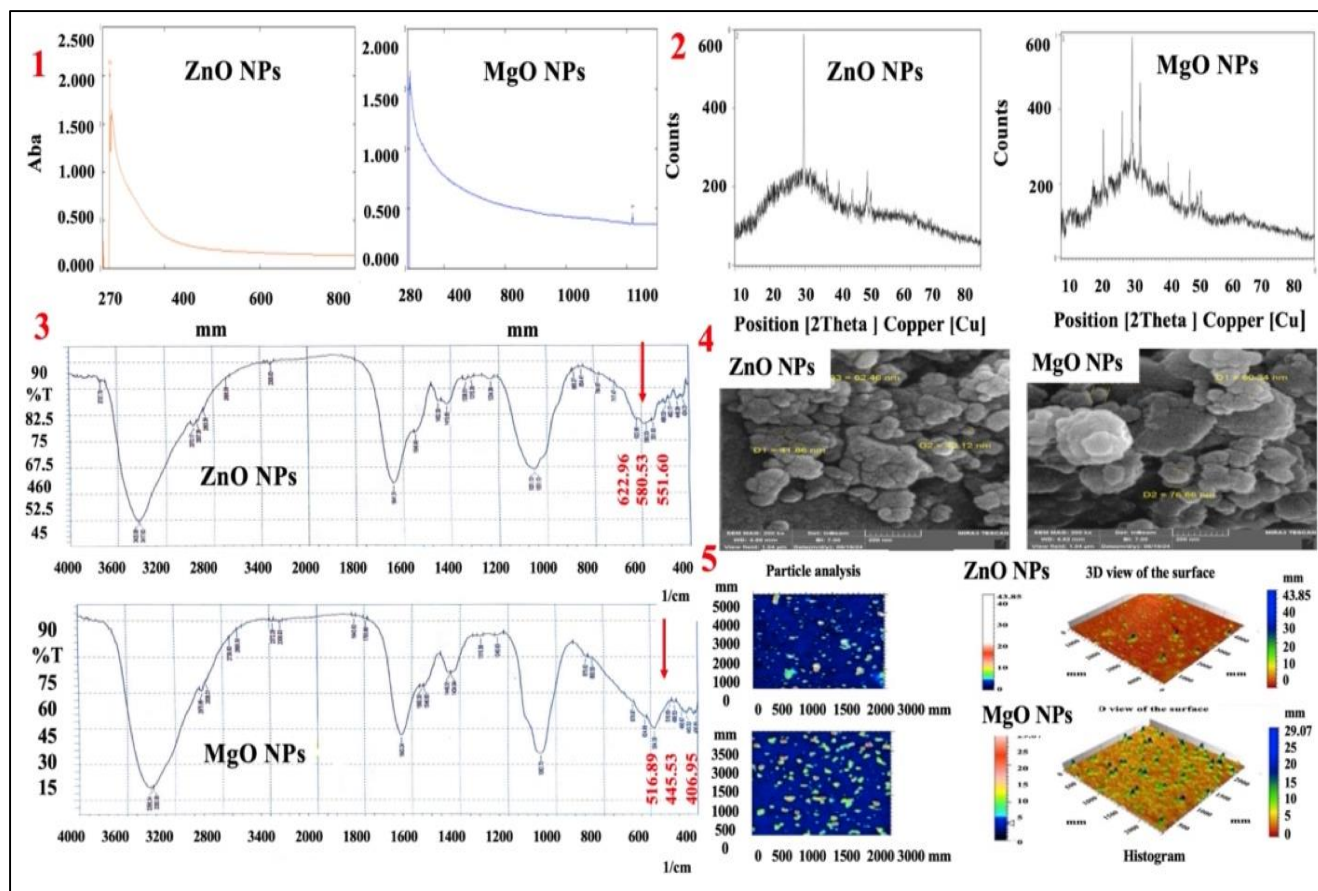


Figure 3. Characterization of ZnO and MgO nanoparticles using various techniques: 1- UV-vis spectra, 2- XRD, 3- FT-IR spectroscopy, 4- EF-SEM, and 5- AFM

X-Ray Diffraction

The diffraction forms of XRD (at 2θ values) showed main peaks of 30, 38, 40, 45, and 48 for ZnO nanoparticles and 22, 27, 30, 32, 40, 45, and 49 for MgO nanoparticles (**Figure 3, part 2**). The observable line broadening in the XRD peaks designated that the synthesized material was in the nm range. This analysis confirmed the successful

formation of high-purity crystalline structures. While the ZnO nanoparticles exhibited the characteristic hexagonal wurtzite phase, the diffractogram of the MgO nanoparticles revealed a face-centered cubic structure.

Fourier Transform Infrared Spectroscopy

The ZnO nanoparticles with their FT-IR spectra are shown in **Figure 3 (part3)**. Functional group bands in the

ZnO nanoparticles were visible at 3433.06, 2970.17, 1641.31, 1452.30, 1051.13, 885.27, 784.97, and 580.53 cm^{-1} . A strong broad band at 3433.06 cm^{-1} resulted from stretching of the hydroxyl groups (O-H). The absorption band seen at 2970.17 cm^{-1} occurred due to vibrational stretching of the CH group. The C=C stretching band vibration could be observed at 1641.31 cm^{-1} . Moreover, 1452.30 cm^{-1} was the -C-H- bending vibration band frequency. The vibrational bending of the =C-H groups was in charge of the peaks at 1051.13 and 885.27 cm^{-1} . The band at 784.97 cm^{-1} could be attributed to the bending vibration of the O-H group. Lastly, the absorption peak of ZnO stretching vibrations was reported at 580.53 cm^{-1} (Figure 3, part 3). Additionally, the FT-IR spectrum of the MgO nanoparticles exhibited a characteristic absorption peak at 875.62 cm^{-1} , which is attributed to the delta (O—C=O) vibrations. This peak indicated the degradation of organic compounds, such as alkanes and aromatics, originally present in the bacterial filtrate during the synthesis process. The formation of MgO nanoparticles was further confirmed by the appearance of intense absorption bands in the low-frequency region. The specific peaks observed at 406.95, 445.53, and 516.89 cm^{-1} (Figure 3, part 3) are characteristics of the Mg-O stretching vibrations, representing the lattice vibrations of the MgO crystalline structure. This phenomenon is likely triggered by the use of magnesium chloride as a precursor. Furthermore, the presence of peaks at 3398.34 cm^{-1} and 1643.24 cm^{-1} corresponded to the stretching and bending vibrations of the O-H groups, respectively, confirming the

role of bioactive molecules in capping and stabilizing the MgO nanoparticles.

Field Emission Scanning Electron Microscopy

The FE-SEM results of the biosynthesized nanoparticles are shown in Figure 3 (part 4), which reveals that both ZnO and MgO nanoparticles are approximately spherical with average diameters of 48.81 nm and 64.55 nm, respectively. In addition, these nanoparticles were aggregated and formed predominantly dense and irregular sharp flakes.

Atomic Force Microscopy

The AFM findings showed that ZnO and MgO nanoparticles were spherical and had two and three dimensions, respectively (Figure 3, part 5). The particles were solitary or aggregated with mean particle sizes of 62.08 nm and 46.43 nm, respectively.

Antibacterial Activity against *P. aeruginosa*

It is clear from Table 1 that the 100 mg/mL concentration of all the tested compounds, except MgO nanoparticles, exerted the highest inhibitory activity on the growth of *P. aeruginosa* isolates compared to the other concentrations. The bacteriocin-ZnO nanoparticles composite exerted higher and significantly ($P < 0.05$) important zones of inhibition than the other compounds, and its impact continued till the concentration 25 mg/mL (Figure 4). Regarding the lowest concentrations of all compounds starting from 12.5 mg/mL to 1.56 mg/mL, they were worthless.

Table 1. Inhibition zones (mm) of *P. aeruginosa* isolates toward bacteriocin, ZnO nanoparticles, MgO nanoparticles, and their composites at different concentrations

Compound	Concentration (mg/mL)			Significant difference
	100	50	25	
Bacteriocin	18.66 ± 0.33 ^{Ba}	11.33 ± 0.33 ^{Ba}	0 ^C	*
ZnO nanoparticles	15.66 ± 0.33 ^{Ca}	13.00 ± 0.57 ^{Ab}	12.66 ± 0.33 ^{Ab}	*
MgO nanoparticles	0.000 ± 0.00 ^E	0.000 ± 0.00 ^C	0.000 ± 0.00 ^C	NS
Bacteriocin + ZnO nanoparticles	21.00 ± 0.57 ^{Aa}	11.00 ± 0.57 ^{Ba}	10.66 ± 0.33 ^{Bb}	*
Bacteriocin + MgO nanoparticles	11.33 ± 0.66 ^D	11.00 ± 0.57 ^B	0.000 ± 0.00 ^C	NS
Significant difference	*	*	*	

*: Significant, NS: Not significant. Different alphabetical small letters (a and b) within the same row indicate the occurrence of significant differences among the various concentrations. While the different capital letters (A to D) within the same column indicate the existence of significant differences among the compounds. Data are shown as a mean ± SD, n = 3

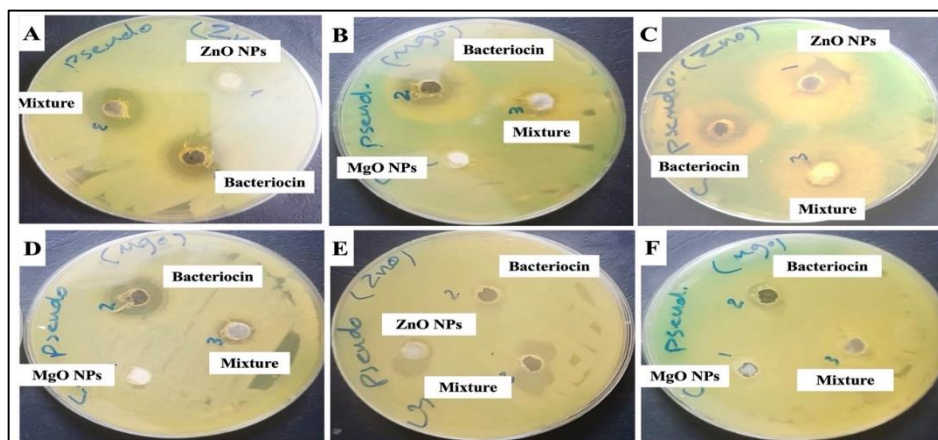


Figure 4. Effects of various concentrations of bacteriocin, ZnO nanoparticles, MgO nanoparticles, and their mixture on the growth of *P. aeruginosa*. A & B- 100 mg/mL, C & D- 50 mg/mL, E & F- 25 mg/mL

Antibacterial Activity against *S. aureus*

The tested substances and their combinations exhibited varying degrees of antibacterial activity, mainly across the three concentrations shown in **Table 2** and **Figure 5**. Statistical analysis displayed significant ($P < 0.05$) differences in the inhibition zones for most treatments, except for MgO nanoparticles. ZnO nanoparticles and their composite with bacteriocin demonstrated the most potent and significant antibacterial effect, with an MIC of 25 mg/mL. Upon their use separately or in combination, bacteriocin and ZnO nanoparticles showed a clear concentration-dependent response. The lowest

concentrations (12.5 to 1.56 mg/mL) of all treatments were ineffective (data not shown).

Antibacterial Activity against *E. coli*

The ZnO nanoparticles exhibited a higher antibacterial activity than bacteriocin, with an MIC value of 50 mg/mL for each. Treating *E. coli* culture with a combination of ZnO nanoparticles and bacteriocin resulted in the significantly ($P < 0.05$) highest inhibition zones (**Table 3, Figure 6**). MgO nanoparticles, on the other hand, demonstrated a complete lack of inhibitory effect at all the concentrations used. The lowest concentrations (12.5 to 1.56 mg/mL) were inefficient (data not shown).

Table 2. Inhibition zones (mm) of *S. aureus* isolates toward bacteriocin, ZnO nanoparticles, MgO nanoparticles, and their combinations

Compound	Concentration (mg/mL)			Significant difference
	100	50	25	
Bacteriocin	25.00 ± 0.57 ^{Ba}	14.33 ± 0.66 ^{Cb}	12.66 ± 0.33 ^{Bb}	*
ZnO nanoparticles	27.66 ± 0.33 ^{Aa}	22.33 ± 0.33 ^{Ab}	19.33 ± 0.66 ^{Ac}	*
MgO nanoparticles	0.000 ± 0.00 ^{Da}	0.000 ± 0.00 ^{Da}	0.000 ± 0.00 ^{Da}	NS
Bacteriocin + ZnO nanoparticles	27.33 ± 1.76 ^{Aa}	24.00 ± 0.57 ^{Aa}	11.33 ± 0.33 ^{Bb}	*
Bacteriocin+ MgO nanoparticles	14.33 ± 0.33 ^{Ca}	11.00 ± 0.57 ^{Cb}	0.000 ± 0.00 ^{Dc}	*
Significant difference	*	*	*	

*, Significant, NS: Not significant. Different alphabetical small letters (a to c) within the same row indicate the existence of significant differences among the various concentrations. While the different capital letters (A to D) within the same column refer to the occurrence of significant differences among the compounds. Data are shown as a mean ± SD, n = 3

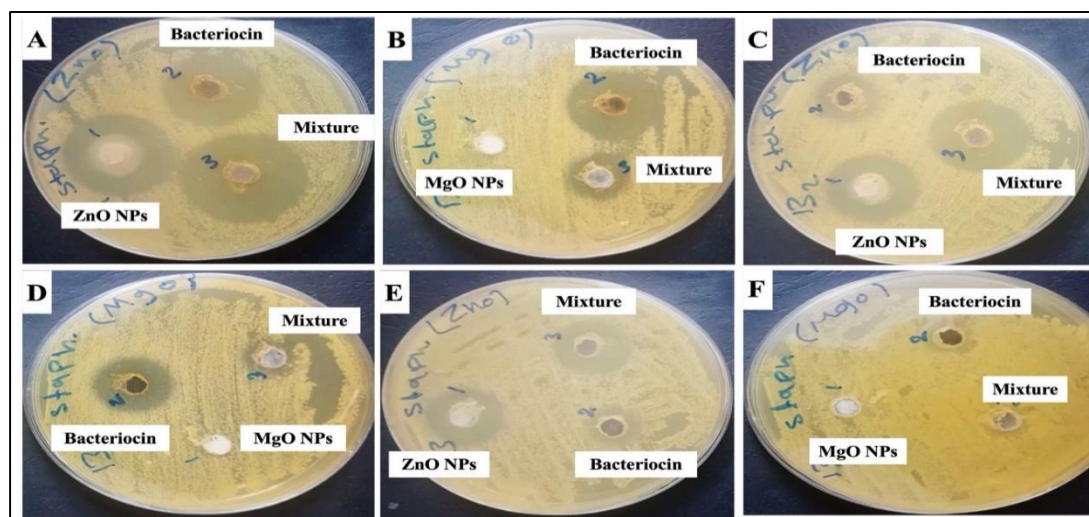


Figure 5. Effects of various concentrations of bacteriocin, ZnO nanoparticles, MgO nanoparticles, and their mixture on the growth of *S. aureus*. A & B- 100 mg/mL, C & D- 50 mg/mL, E & F- 25 mg/mL

Table 3. Inhibition zones (mm) of *E. coli* isolates toward bacteriocin, ZnO nanoparticles, MgO nanoparticles, and their combinations

Compound	Concentration (mg/mL)			Significant difference
	100	50	25	
Bacteriocin	15.00 ± 1.54 ^B	13.66 ± 0.33 ^B	0.000 ± 0.00	NS
ZnO nanoparticles	22.66 ± 0.33 ^{Aa}	18.66 ± 0.33 ^{Ab}	0.000 ± 0.00	*
MgO nanoparticles	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	NS
Bacteriocin + ZnO nanoparticles	23.00 ± 0.57 ^{Aa}	19.33 ± 0.66 ^{Ab}	0.000 ± 0.00	*
Bacteriocin + MgO nanoparticles	12.66 ± 0.33 ^{Ca}	9.66 ± 0.33 ^{Cb}	0.000 ± 0.00	*
Significant difference	*	*	NS	

*, Significant, NS: Not significant. Different alphabetical small letters (a and b) within the same row signify the occurrence of significant differences among the various concentrations. While the different capital letters (A to C) within the same column denote the incidence of significant differences among the compounds. Data are shown as a mean ± SD, n = 3

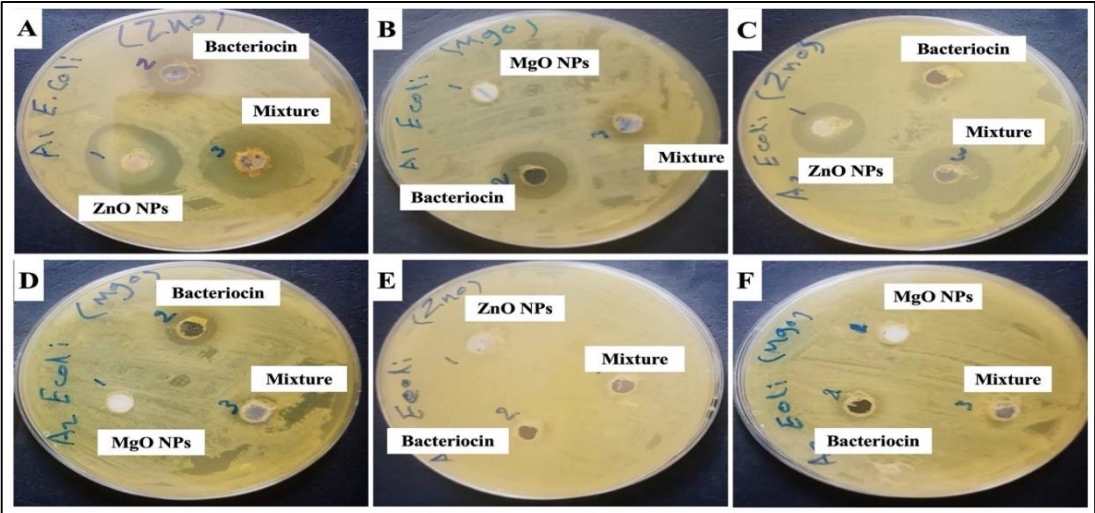


Figure 6. Effects of various concentrations of bacteriocin, ZnO nanoparticles, MgO nanoparticles, and their mixture on the growth of *E. coli*. A & B- 100 mg/mL, C & D- 50 mg/mL, E & F- 25 mg/mL

Table 4. Inhibition zones (mm) of *Bacillus* spp. isolates toward bacteriocin, ZnO nanoparticles, MgO nanoparticles, and their combinations

Compound	Concentration (mg/mL)						Significant difference
	100	50	25	12.5	6.25	3.125	
Bacteriocin	23.0 ± 0.57 ^B	22.7 ± 1.52 ^B	0.00 ± 0.00 ^B	0.00 ± 0.00 ^B	0.00 ± 0.00 ^B	0.00 ± 0.00 ^B	NS
ZnO nanoparticles	28.7 ± 0.66 ^{Aa}	26.0 ± 0.57 ^{Ab}	24.0 ± 0.57 ^{Ab}	19.0 ± 0.57 ^{Ac}	11.7 ± 0.66 ^{Ad}	10.3 ± 0.88 ^{Ad}	*
MgO nanoparticles	9.33 ± 0.33 ^{Da}	0.00 ± 0.00 ^C	0.00 ± 0.00 ^B	0.00 ± 0.00 ^B	0.00 ± 0.00 ^B	0.00 ± 0.00 ^B	*
Bacteriocin + ZnO nanoparticles	29.0 ± 0.57 ^{Aa}	27.3 ± 0.33 ^{Ab}	24.0 ± 0.57 ^{Ac}	20.7 ± 0.33 ^{Ad}	0.00 ± 0.00 ^B	0.00 ± 0.00 ^B	*
Bacteriocin + MgO nanoparticles	15.3 ± 0.33 ^{Ca}	0.00 ± 0.00 ^C	0.00 ± 0.00 ^B	0.00 ± 0.00 ^B	0.00 ± 0.00 ^B	0.00 ± 0.00 ^B	*
Significant difference	*	*	*	*	*	*	

*: Significant, NS: Not significant. Different alphabetical small letters (a to d) within the same row denote the existence of significant differences among the various concentrations. While the different capital letters (A to D) within the same column mean the occurrence of significant differences among the compounds. Data are shown as a mean ± SD, n = 3

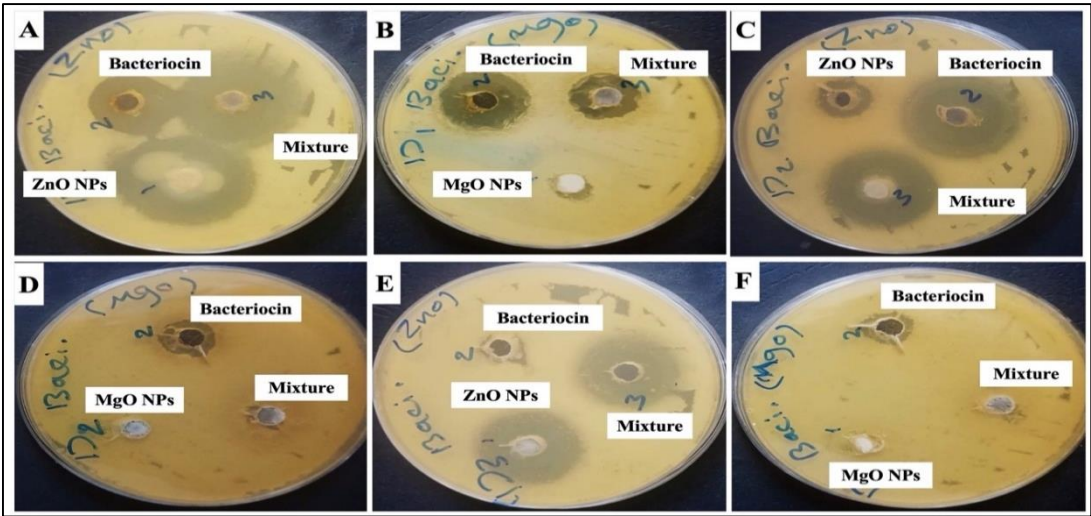


Figure 7. Effects of various concentrations of bacteriocin, ZnO NPs, MgO NPs, and their mixture on the growth of *Bacillus* spp. A & B- 100 mg/mL, C & D- 50 mg/mL, E & F- 25 mg/mL

Antibacterial Activity against *Bacillus* spp.

The antibacterial activity against *Bacillus* spp., shown in **Table 4** and **Figure 7**, reveals varying degrees of efficiency across the tested compounds. Importantly, the ZnO nanoparticles demonstrated significantly ($P < 0.05$) higher

inhibition zones than bacteriocin. Additionally, these nanoparticles exerted the most consistent concentration-dependent response, maintaining a significant inhibitory activity up to the concentration 3.125 mg/mL. Adding ZnO nanoparticles to bacteriocin increased the leverage of growth inhibition, broadening the impact till 12.5 mg/mL

compared to the use of bacteriocin. Similarly, bacteriocin plus MgO composites showed an improved response compared to the individual use of MgO nanoparticles.

According to the findings illustrated above, it appears that *S. aureus* followed by *Bacillus* spp. were the most affected by bacteriocin, ZnO nanoparticles, and the nanocomposites, in a dose-dependent concentration.

DISCUSSION

Prior to bacteriocin production from *L. acidophilus*, the identity of this bacterium was successfully confirmed through the PCR assay and the 16S rRNA gene sequencing, whose nucleotide sequence was registered under the accession number PZ389740. After that, various conditions were tested in the present study to optimize the bacteriocin production. Temperature, pH, and nutrient availability have been shown to have a crucial role in bacteriocin synthesis (6,26). Upon investigating the impact of culture media, the data of the current study is consistent with that of Mahdy et al. (27), who also evaluated various media for bacteriocin production from *Lactobacillus helveticus* strain DF, and observed that the MRS medium provided a more complex nitrogen source and growth factors necessary for the induction of bacteriocin synthesis. The results of this study regarding the pH effect also align with those of Juárez Tomás et al. (28), who indicated that the largest inhibitory activity of bacteriocin was achieved at pH 6.0. However, the opposite to this study findings was observed by Hasan et al. (6), who stated that the best effect and stability of bacteriocin stemmed from *L. acidophilus* occurred at pH 5 and temperature 30°C. Optimal conditions for bacteriocin production have been verified to vary among *Lactobacillus* spp. (29,30).

With respect to the nanoparticles synthesis, a primary point of divergence of this study from other studies in Iraq, is the green synthesis of ZnO and MgO nanoparticles, utilizing the cell-free filtrate of *E. coli* as a stabilizing and reducing agent. This eco-friendly method stands in contrast to the chemical synthesis routes commonly documented in local literature, offering a less toxic and more biocompatible alternative. In a contemporary nanotechnology, the easy and quick multiplication of microorganisms is becoming more attractive (16). Only a few studies have been conducted to synthesize ZnO nanoparticles by microorganisms. Microbial-mediated production of nanoparticles, however, is still a promising but little studied technology (16). The current research confirms the formation of pure nanostructures, whose features are also compatible with what was documented by other researchers (31-37).

Pertaining to the antibacterial effect of the diverse compounds applied against different food-borne bacteria, the 100 mg/mL concentration had higher antibacterial effects than the other concentrations. The use of bacteriocin alone had a great antibacterial effect toward all the tested pathogens, with the strongest inhibitory influence was reported in *S. aureus*. Bacteriocins generated by *L. acidophilus* have shown strong antibacterial action against many pathogenic and food spoilage microbes (29).

Bacteriocins of this bacterium are antimicrobial peptides, which inhibit or kill pathogenic bacterial strains, e.g., *Listeria monocytogenes*, *S. aureus*, *E. coli*, and *Salmonella* spp. (38,39). It has been demonstrated that the bacteriocin derived from *L. acidophilus* exhibits a broad-spectrum inhibitory effect against various pathogenic bacteria due to its ability to induce pore formation in the bacterial cytoplasmic membrane, leading to the escape of vital intracellular components (40).

Concerning the nanomaterials, MgO nanoparticles did not reveal any inhibitory activity, even at high concentrations, toward the tested bacteria. This observation is compatible with that of Hashemi et al. (41) who have recently found that the antimicrobial efficiency of MgO nanoparticles is often limited because of their low solubility and the specific kinetics of ion release. However, these nanoparticles have been indicated to possess antibacterial properties with a significant microbial inhibition when they are used at high concentrations (42). On the other hand, ZnO nanoparticles manifested a considerable antibacterial activity against all the examined isolates, and the microbial growth inhibition is a concentration-dependent. This is consistent with previous findings (43,44). Additionally, the inhibition zones of these nanoparticles at various concentrations were extremely bigger than those of the bacteriocin toward the same bacteria tested herein. *Bacillus* spp. was the most susceptible to the ZnO nanoparticles followed by *S. aureus*, although a considerable growth inhibition occurred in case of *P. aeruginosa* and *E. coli*. Gram-positive bacteria have been found to be more susceptible than Gram-negative ones to ZnO nanoparticles (45,46). The antibacterial efficiency of ZnO nanoparticles was reported against food-related pathogens, e.g., *E. coli*, *Pseudomonas fluorescens*, and *Bacillus subtilis* (47). Many studies have documented the effective role of ZnO nanoparticles on various microorganisms. For instance, the ZnO nano-fluids exerted bacteriostatic effects on *E. coli* O157:H7 by causing cell membrane damages (48). They have also exerted excellent bactericidal effect against *E. coli*, *S. aureus*, and *Bacillus atrophaceus* (49-51). Moreover, the ZnO nanoparticles had antibacterial impacts toward *S. Enteritidis* and *L. monocytogenes* (52). The antibacterial properties of the ZnO nanoparticles have been noticed to increase upon increasing the surface area and decreasing the size of their particles (53). Furthermore, the spherical morphology of these particles facilitates their attachment and subsequent penetration into the bacterial cell wall, resulting in irreversible membrane injury (54). These nanoparticles have also been suggested to possess numerous mechanisms to exert their antimicrobial effects (not within the scope of this study).

When comparing the zones of inhibition caused by bacteriocin alone, ZnO nanoparticles alone, and the bacteriocin-ZnO composite, it seems that the inhibitory activity against the bacteria, regardless of its type, has augmented upon mixing bacteriocin with the ZnO nanoparticles. It has been reported that following conjugation of bacteriocins with nanoparticles, the

antibacterial activity against spoilage bacteria is increased (55). Integration of gold nanoparticles with a bacteriocin released from *L. acidophilus* CH1 has resulted in a formulation with a robust effect on immunocompromised mice with intestinal microsporidiosis (56). These metal nanoparticle-bacteriocin conjugates inhibit bacterial growth through a synergistic, multi-targeted approach, combining the structural disruption caused by bacteriocins with the membrane damage and oxidative stress induced by metal ions. While bacteriocins form pores in the membrane, the nanoparticles generate ROS, leading to cellular content leakage, DNA damage, and protein dysfunction (57,58). With respect to bacteriocin-MgO composite, the current study showed an inhibitory effect against *Bacillus* spp. only at the highest concentration. In a similar context, the experimental data of Jin and He, (59) revealed that the integration of nisin with MgO nanoparticles significantly improved the antibacterial activity in comparison to MgO nanoparticles used alone.

The novelty of this study, in comparison to the existing research conducted in Iraq, lies in its integrative approach toward antimicrobial synergy. While the existing local studies in Iraq predominantly focus on the inhibitory effects of individual agents, such as ZnO nanoparticles (60), MgO nanoparticles (61), or bacteriocins (62). Globally, the scientific community is shifting away from the monotherapy due to the rapid occurrence of multidimensional microbial resistance. By bridging the gap between the nanotechnology and natural antimicrobial peptides (bacteriocins), this research not only transcends the limitations of local individual-agent studies but also contributes to the global effort for developing potent, synergistic formulations that lower the required therapeutic doses and minimize the risk of further resistance development (63). Up to our knowledge, no literature online has used the same combination of bacteriocin from *L. acidophilus* with ZnO or MgO nanoparticles produced by *E. coli* against the food-borne pathogens *S. aureus*, *P. aeruginosa*, *E. coli*, and *Bacillus* spp. Despite the promising findings gained from the present investigation, some limitations are still existed, such as detecting *Bacillus* to the species level, the bacteriocin's mechanisms of action, as well as the in-vivo efficacy and cytotoxicity of the nanoparticles. In conclusion, this study demonstrates that combining *L. acidophilus* bacteriocin with ZnO nanoparticles significantly enhances antibacterial efficacy against broad-spectrum foodborne pathogens. This dual-action mechanism effectively overcomes microbial resistance while addressing the limitations of single-agent treatments, particularly the narrow spectrum of bacteriocins and the potential toxicity of high-dose metal nanoparticles.

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N/A.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

I.J.L. designed and conceptualized the study, wrote and edited the final manuscript, and analyzed the data. E.F.H. performed the laboratory work and wrote the first draft of the manuscript. S.A.L. reviewed and edited the final version of the manuscript. All authors have read and approved the last manuscript.

ARTIFICIAL INTELLIGENT DECLARATION

The authors declare that they are responsible for the accuracy and integrity of all content of the manuscript, including part generated by AI, and it is not used as a co-author.

EDITORIAL TRANSPARENCY

Inam J Lafta serves as a member of the editorial board for The Iraqi Journal of Veterinary Medicine. Despite this role, the peer review process and the final publication decision were made independently and impartially, ensuring no influence from the author's editorial position.

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الفعالية المختبرية لمركبات البكتيريوسين النانوية ضد الجراثيم الملوثة للأغذية

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الخلاصة

يشهد المجتمع العلمي حالياً تحولاً جذرياً نحو دمج تقنية النانو مع المكونات البيولوجية لمواجهة التحديات المتزايدة لمقاومة الميكروبات. ولذلك، هدفت هذه الدراسة إلى تحضير تركيبات مختلفة من مركبات البكتيريوسين النانوية ودراسة نشاطها المضاد لبعض الجراثيم المنقولة بالغذاء. في هذه الدراسة، جرى مقارنة واختيار الظروف الأفضل لإنتاج كمية أكبر من البكتيريوسين من جرثومة *Lactobacillus acidophilus* في بيئات زرعية ودرجات حرارة ومستويات حموضة مختلفة، تلاها تنقية جزئية باستخدام كروماتوغرافيا الترشيح الهلامي. في الوقت نفسه، صنعت جسيمات نانوية من أكسيد الزنك وأكسيد المغنيسيوم باستخدام طريقة التخليق الحيوي خارج الخلية، إذ جرى توصيف هذه المركبات باستخدام تقنيات حيود الأشعة السينية (XRD) والأشعة تحت الحمراء (FT-IR) والأشعة فوق البنفسجية المرئية (UV-Vis) والمجهر الإلكتروني الماسح ذي الانبعاث الميداني (FE-SEM) والمجهر الذري الماسح (AFM) لتأكيد خصائصها البنيوية. أخيراً، جرى اختبار الفعالية التثبيطية بعدة تراكيز (للبكتيريوسينات والجسيمات النانوية وتركيباتها المختلفة في المختبر باستخدام طريقة الانتشار في الحفر على الأكار ضد بعض مسببات الأمراض المنقولة بالغذاء. أظهرت النتائج أن الظروف المثلى لإنتاج البكتيريوسين تحققت في مرق دي مان-روجوزا (MRS) عند درجة حرارة 37°م ودرجة حموضة 6.0، وكشفت دراسة خصائص جسيمات أكسيد الزنك وأكسيد المغنيسيوم النانوية عن أشكال كروية وتجمعات مفردة بمتوسط أحجام 62.08 و46.43 نانومتر، على التوالي. ومن المثير للاهتمام أن الاستخدام المشترك للبكتيريوسينات وجسيمات أكسيد الزنك النانوية أظهر تأثيرات ملحوظة مضادة للجراثيم، تمثلت في مناطق تثبيط أوسع (23، 30، 20، 30 ملم) ضد جراثيم الإشريكية القولونية، والمكورات العنقودية الذهبية، والزائفة الزنجارية، وجنس الجراثيم العصوية، على التوالي، مقارنةً باستخدام كل منهما على حدة. ويمكن الاستنتاج أن دمج جسيمات أكسيد الزنك النانوية مع البكتيريوسينات المحسنة يعزز بشكل كبير قدرتها المضادة للجراثيم المسببة للأمراض المنقولة بالغذاء. تشير هذه النتائج إلى أن هذا المزيج قد يُشكل نظاماً واعداً ومستداماً لحفظ الأغذية بيولوجياً.

الكلمات المفاحية: بكتيريوسين، جسيمات أكسيد الزنك النانوية، جسيمات أكسيد المغنيسيوم النانوية، طريقة صديقة للبيئة، الفعالية المضادة للجراثيم، مسببات الأمراض المنقولة بالغذاء