



Article

# Green Synthesis of Zinc Oxide Nanoparticles Mediated By Lacticaseibacillus Rhamnosus: Evaluation of Their Antioxidant and Hemolytic Activities

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**Abstract:** Using probiotics to biosynthesize metal oxide nanoparticles is a new, more biocompatible, and environmentally harmless approach compared to traditional physical and chemical synthesis methods. In the current study, we isolated the probiotic Lacticaseibacillus rhamnosus from local dairy products. Instead of directly using the living cells of this bacterium, we extracted its cell-free extracellular supernatant, the clear liquid remaining in the upper layer of the culture medium after the bacterium's metabolism, and successfully synthesized zinc oxide nanoparticles (ZnO-NPs). The first sign that confirmed the successful synthesis was a distinct color change visible to the naked eye. After preliminary confirmation that the synthesis was complete, we conducted a comprehensive and rigorous set of tests on the physical and chemical properties of these nanoparticles. After observation using Field Emission Scanning Electron Microscopy (FE-SEM), it was found that the synthesized nanoparticles were nearly spherical, with an average diameter of 41.5 nanometers, and the size of all particles fell within the range of 27 to 56 nanometers. The XRD analysis confirmed the crystalline nature of the biosynthesized ZnO nanoparticles, with an average crystallite size of 22.13 nm calculated by Scherrer's equation. FTIR spectrum showed a characteristic Zn–O stretching vibration at 563 cm<sup>-1</sup> and the bands observed in the range of 1448–1580 cm<sup>-1</sup> were attributed to carboxylate and amide II groups, indicating the possible involvement of bacterial biomolecules in the capping of nanoparticles. AFM images showed the vertical height values from –0.726 nm to 0.920 nm and the peak to valley height difference of 1.646 nm. The nanoparticles showed strong DPPH radical scavenging activity, and it was found to be concentration-dependent, with 91.89% inhibition at 1000 µg/mL. Moreover, in vitro hemolysis assays with human erythrocytes showed 0% hemolysis at concentrations of 125–500 µg/mL and 1.60% at 1000 µg/mL, indicating low hemolytic activity under the tested in vitro conditions. The present results suggest that the synthesized ZnO nanoparticles derived from L. rhamnosus could be potential potent and non-toxic candidates for biomedical and pharmaceutical applications.

**Keywords:** Green synthesis; Zinc oxide nanoparticles; lacticaseibacillus rhamnosus; Antioxidant activity; Hemolysis

## 1. Introduction

Nanotechnology is a field of science developed in the twenty-first century, fundamentally driven by the reduction of matter to the nanometer scale, typically between 1 and 100 nm. Nanoparticles (NPs) exhibit unique physicochemical behaviors and elevated

surface-to-volume ratios that drastically differ from their bulk counterparts [1]. The application including materials manufacturing, agriculture, environmental science, and biomedical therapeutics, ranging from advanced diagnostics to targeted cancer therapies. Generally, nanotechnology has two synthetic strategies: top-down and bottom-up approaches [2], [3]. The top-down methodology relies on physical and mechanical techniques such as electro-explosion, milling, etching, lithography, and laser ablation to break down bulk materials [4]. While the bottom-up approach occurs through chemical or biological assembly of atomic and molecular components, conventional physical and chemical protocols are frequently constrained by high energy consumption, prohibitive operational costs, and the generation of hazardous, ecologically toxic chemical byproducts [5]. The green synthesis of NPs has scientific interest as a biocompatible, cost-effective, non-toxic, and eco-friendly alternative [6].

Among the biological matrices investigated for biogenic fabrication, beneficial food-related microorganisms, predominantly lactic acid bacteria (LAB), yeasts, and acetic acid bacteria (AAB), were prominent green nanofactories [7]. LAB are Gram-positive, catalase-negative, acid-tolerant, non-motile, aerotolerant, and non-sporulating microorganisms that primarily generate lactic acid via carbohydrate fermentation [8]. This group includes several genera of ecological and industrial importance, such as *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc* and *Weissella* [9]. That is, live microorganisms which, when administered in adequate amounts, confer a health benefit on the host <sup>(14, 10)</sup>. Because of their probiotic status, LAB have branched out into essential pillars of modern biotechnology and nanomedicine [10].

The use of LAB in green synthesis not only provides a sustainable fabrication pipeline but also heavily coats the resultant NPs with bioactive cellular components, which improves their stability, biocompatibility and therapeutic profiles [11]. Structurally, LAB have thick and strong cell walls containing glycosides, peptides, polysaccharides and proteins. These macromolecular scaffolds carry a net negative electrical potential, which makes them natural sites for bioreduction and nucleation of metal ions, leading to the formation of nanoparticles and protection of bacterial cells from heavy metal toxicity [12]. Thus, different LAB strains isolated from functional foods have been successfully employed to synthesize metallic and metalloid nanoparticles, such as silver (Ag), selenium (Se), gold (Au), and zinc oxide (ZnO) [13].

Among the various metal oxides, zinc oxide nanoparticles (ZnO-NPs) have gained prominence in the past two decades due to their excellent biocompatibility, economic feasibility, and low systemic toxicity in biomedical applications [14]. Biogenic ZnO-NPs possess excellent luminescent properties useful in bioimaging, exhibit significant antidiabetic effects by preserving the structural integrity of insulin, and are able to selectively induce apoptosis in damaged cellular pathways [15]. Furthermore, the diverse physical shapes and sizes of synthesized ZnO-NPs grant them vast functional adaptability across target-specific drug delivery and therapeutic interventions [16]. The optimization of these nanomaterials is highly sensitive to strain-specific metabolic differences and precise extraction microenvironments <sup>(26)</sup>. Despite the extensive use of LAB strains in nanobiotechnology, the specific use of the robust probiotic *L. rhamnosus* as a stabilizer/reducer of ZnO-NPs has not been explored, especially in terms of the influence of its specific exopolysaccharide and surface protein profile on the biological safety and radical scavenging dynamics of the final product [17].

Although many studies have demonstrated the green synthesis of zinc oxide nanoparticles (ZnO-NPs) using different plants, fungi, and various bacterial species, only a few have reported probiotic bacteria as biological nano factories, particularly *L. rhamnosus* [18]. In addition, previous works mostly focused on the synthesis of nanoparticles and their basic physicochemical characterizations, and there are still limited comprehensive investigations that combine structural characterization with biological

evaluation (e.g., antioxidant activity and hemocompatibility). Moreover, the physicochemical and biological properties of ZnO-NPs could be deeply affected by the bacterial strains and synthesis conditions, which requires the identification of efficient probiotic isolates capable of synthesizing biocompatible nanoparticles with enhanced functional properties [19], [20]. Thus, the present study was aimed at the biosynthesis of ZnO-NPs by *L.rhamnosus*, characterization using complementary analytical techniques and assessment of antioxidant potential and hemolytic safety to explore their potential suitability for future biomedical applications [21].

## 2. Methodology

### Bacterial Isolation and Culturing

Dairy products (yogurt, cheese and milk) samples were collected from four local markets in AL Najaf city and transferred to the laboratory in a refrigerated box for isolation and identification. 10 g of each sample was aseptically suspended in 90 mL of sterile 0.1% peptone water to give a  $10^{-1}$  dilution. Serial dilutions were then prepared down to  $10^{-6}$ . Appropriate dilutions (0.1 ml) were plated on sterile MRS agar plates (De Man, Rogosa and Sharpe medium). The medium was supplemented with cycloheximide 0.1 g/L to inhibit fungal growth. Then the plates were incubated anaerobically for 24 h at 37°C.

### Identification of Isolates

Bacterial identification was confirmed with morphological evaluation, Gram stain microscopic evaluation, and VITEK-2 compact system. Single colonies of *L.rhamnosus* were inoculated in MRS agar and anaerobically incubated at 37°C for 24 hours. The resultant cultures were evaluated for specific colony parameters such as the structural form, overall size, surface texture and margin organization.

Definitive identification to species level was performed with the automated VITEK-2 Compact System. Bacterial suspensions were adjusted in 3 mL of sterile saline to a McFarland turbidity standard of 0.5 in accordance with the established protocol of the VITEK 2 Compact System.

### Screening and Selection of Efficient Isolates for ZnO-NPs Synthesis

The bacterial isolates derived from dairy products were screened to determine their capacity for mediating the biogenic fabrication of zinc oxide nanoparticles (ZnO-NPs). The isolates were initially grown in MRS broth anaerobically at 37°C for 24 hours. Following incubation, the bacterial cultures were centrifuged at 4500 rpm for 10 min at 4°C. Then take the cell-free supernatant was taken for extracellular production.

To evaluate extracellular nanoparticle synthesis, a 5 mL of cell free supernatant was treated with 0.01 mg of zinc acetate  $\text{Zn}(\text{CH}_3\text{COO})_2$ . The mixture was incubated in a shaking incubator at 37°C and 100 rpm for 24 hours.

### Large-Scale Production of Biogenic ZnO-NPs

The production of ZnO-NPs using *L. rhamnosus* by modified technique as overnight Individual culturing colonies on MRS agar at culture flask containing 1000 mL of MRS with bacterial isolate was centrifuged for take the cell free supernatant for used in large scale production of ZnO by mix the supernatant with 2 mg of zinc acetate and incubated at 37°C for 24 hours under continuous agitation at 100 rpm, The biogenic product was washed 3 times and centrifuged via a broad-scale refrigerated centrifuge operated at 4 °C with the power 4500xg to pellet the material, after that the remaining microbial cell supernatant was discarded, and the gathered pellet was collected into sterile glassware. Finally, the collected wet precipitate was placed in a drying oven.

### Physical Characterization of Biosynthesized ZnO-NPs

The baseline structural, morphological, and optical characteristics of the biosynthesized ZnO-NPs were extensively elucidated utilizing advanced analytical

techniques. The size estimation and formation of the ZnO nanoparticles within the composite suspension were confirmed by UV-Visible spectroscopy (Shimadzu UV-Visible 1800). Field Emission Scanning Electron Microscopy (FESEM) and Atomic Force Microscopy (AFM) were used to resolve detailed surface topography, physical distribution, and morphology. X-ray diffraction (XRD) and Fourier-Transform Infrared Spectroscopy (FTIR) were used at the study AL-Ameen laboratory of Imam Ali Holy Shrine to characterize the particular crystal framework and chemical functional groups. correspondingly, using the methods described in.

#### Evaluation of Antioxidant Activity

A conventional 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable free-radical scavenging experiment was used to assess the biogenic "ZnO-NPs" capacity to scavenge radicals *in vitro*. A sterile 96-well microplate was used to conduct the reaction. 100  $\mu\text{L}$  of the DPPH working reagent was thoroughly mixed with 100  $\mu\text{L}$  of the produced "ZnO-NPs" suspension at different gradient concentrations (1000, 500, 250, and 125  $\mu\text{g/mL}$ ). For the baseline controls, 200  $\mu\text{L}$  of 96% methanol was placed to the microplate's first well as a negative control, and 200  $\mu\text{L}$  of pure DPPH reagent was applied to the designated positive control well. To enable steady-state radical scavenging, the microplate was incubated for 30 minutes at room temperature in total darkness. An automated ELISA microplate reader was used to record the final post-incubation absorbance profile at 514 nm.

The following formula was used to determine the DPPH radical scavenging activity:

$$\text{DPPH inhibition (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100,$$

where  $A_{\text{control}}$  is the absorbance of the DPPH solution without ZnO-NPs and  $A_{\text{sample}}$  is the absorbance of the DPPH solution with ZnO-NPs.

#### Hemolytic Activity Profiling

To detect the systemic biocompatibility of the synthesized nanomaterials, an *in vitro* hemolysis assay was performed utilizing whole blood gathered from a healthy donor. Immediately in sterile Eppendorf tubes, a baseline reaction volume was established consisting of 285  $\mu\text{L}$  of whole blood. To this, 15  $\mu\text{L}$  of biogenic ZnO-NPs at (1000, 500, 250, and 125  $\mu\text{g/mL}$ ) concentrations were introduced. For assay validation, a positive control representing 100% blood lysis was prepared by treating blood with 15  $\mu\text{L}$  of Triton X-100, while a negative control representing 0% baseline lysis was prepared by adding 15  $\mu\text{L}$  of sterile Phosphate-Buffered Saline (PBS). The reaction vessels were incubated on a platform shaker at 37°C and 90 rpm for a duration of 4 hours. Following the exposure period, the tubes were centrifuged at 10,000 rpm for 10 minutes to pellet non-lysed red blood cells. A 100  $\mu\text{L}$  aliquot of the resulting supernatant from each tube was transferred to a microplate, and the free hemoglobin release was quantified by tracking absorbance at 550 nm via an ELISA scanning spectrophotometer system.

The percentage of hemolysis was calculated using the following equation: Hemolysis (%) =  $[(A_{\text{sample}} - A_{\text{negative control}}) / (A_{\text{positive control}} - A_{\text{negative control}})] \times 100$ . Negative calculated values were considered 0% after baseline correction.

### 3. Results

#### Selection and identification of Biosynthetic Probiotic Isolates

The selection of the most efficient nanoparticle-producing isolate was initially based on the visual alteration in the reaction mixture after exposure to zinc ions and UV spectroscopic analysis [22]. The observed transition from a light color to a darker appearance was considered a preliminary indication of ZnO nanoparticle formation, as shown in Figure 1 [23].

The selected isolate was identified using the VITEK 2 Compact system, as shown in Figure 2.



**Figure 1.** Large scale production of ZnO nanoparticles by *L. rhamnosus*

bioMérieux Customer: Microbiology Chart Report Printed March 14, 2026 10:30:23 AM AST

Patient Name: AFRS 367 Patient ID:  
 Location: Physician:  
 Lab ID: 2 Isolate Number: 1

Organism Quantity:  
 Selected Organism : *Lactobacillus rhamnosus*

Source: Collected:

|           |  |  |  |  |  |  |  |  |  |  |
|-----------|--|--|--|--|--|--|--|--|--|--|
| Comments: |  |  |  |  |  |  |  |  |  |  |
|           |  |  |  |  |  |  |  |  |  |  |
|           |  |  |  |  |  |  |  |  |  |  |

|                            |  |                                                |  |               |  |
|----------------------------|--|------------------------------------------------|--|---------------|--|
| Identification Information |  | Analysis Time: 6.27 hours                      |  | Status: Final |  |
| Selected Organism          |  | 97% Probability <i>Lactobacillus rhamnosus</i> |  |               |  |
| ID Analysis Messages       |  | Bionumber: 2137710030401                       |  |               |  |

| Biochemical Details |       |   |    |       |   |    |       |   |    |       |   |    |       |   |    |       |   |
|---------------------|-------|---|----|-------|---|----|-------|---|----|-------|---|----|-------|---|----|-------|---|
| 4                   | dGAL  | + | 5  | LeuA  | + | 6  | ELLM  | - | 7  | PheA  | + | 8  | ProA  | + | 10 | PyrA  | - |
| 11                  | dCEL  | + | 13 | TyrA  | + | 15 | APPA  | + | 18 | dGLU  | + | 20 | dMNE  | + | 22 | dMAL  | + |
| 28                  | SAC   | + | 30 | ARB   | + | 33 | NAG   | + | 34 | BGLUI | - | 36 | URE   | - | 37 | BGURI | - |
| 39                  | BGALI | - | 41 | AARA  | - | 42 | AGALI | - | 43 | BMAN  | - | 44 | ARG   | - | 45 | PVATE | - |
| 51                  | MTE   | + | 53 | ESC   | + | 54 | BdFUC | - | 55 | BNAGI | - | 56 | AMANI | - | 57 | AIFUC | - |
| 59                  | PHOS  | - | 60 | IARA  | - | 61 | dRIB2 | - | 62 | OPS   | - | 63 | AARAF | - | 64 | dXYL  | - |
|                     | GRAM  | + |    | MORPH | - |    | AERO  | - |    |       |   |    |       |   |    |       |   |

**Figure 2.** Vitek-2 Compact system identification

### Physicochemical Characterization of Biosynthesized ZnO-NPs

#### 1-UV spectroscopic analysis

The UV-Vis absorption spectrum of the synthesized zinc oxide nanoparticles (ZnO NPs) shows characteristic optical properties typical of nanoscale semiconductor materials [24]. In the ultraviolet region, a well-defined absorption band is observed with a maximum at about 320-350 nm and an absorbance intensity of about 0.37, as shown in Figure 3 [25]. The absorbance falls off sharply in the visible region of the spectrum (400-900 nm) and stays flat (0.18-0.24).

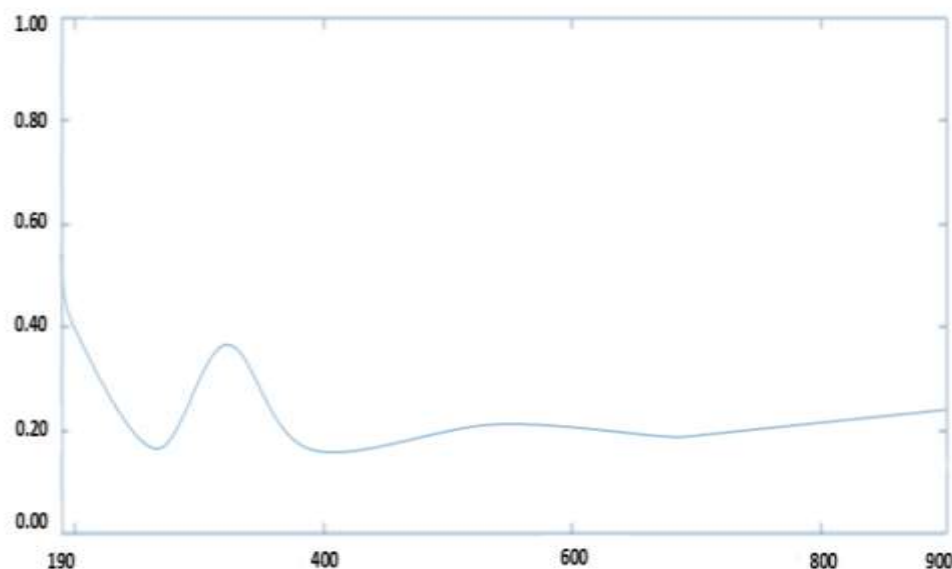


Figure 3. UV Spectroscopic analysis of biosynthesized ZnO nanoparticles

## 2- Morphological Analysis via Field Emission Scanning Electron Microscopy (FESEM)

The FE-SEM micrographs (Figure 4) revealed that the biosynthesized ZnO nanoparticles produced by *L. rhamnosus* were predominantly spherical to quasi-spherical [26]. However, the nanoparticles tended to form dense, porous agglomerates in the normal state, a common feature of nanoscale metal oxide nanoparticles [27]. The particle size analysis showed that the ZnO nanoparticles synthesized were distributed in the range of 27–56 nm with an average particle size of about 41.5 nm.

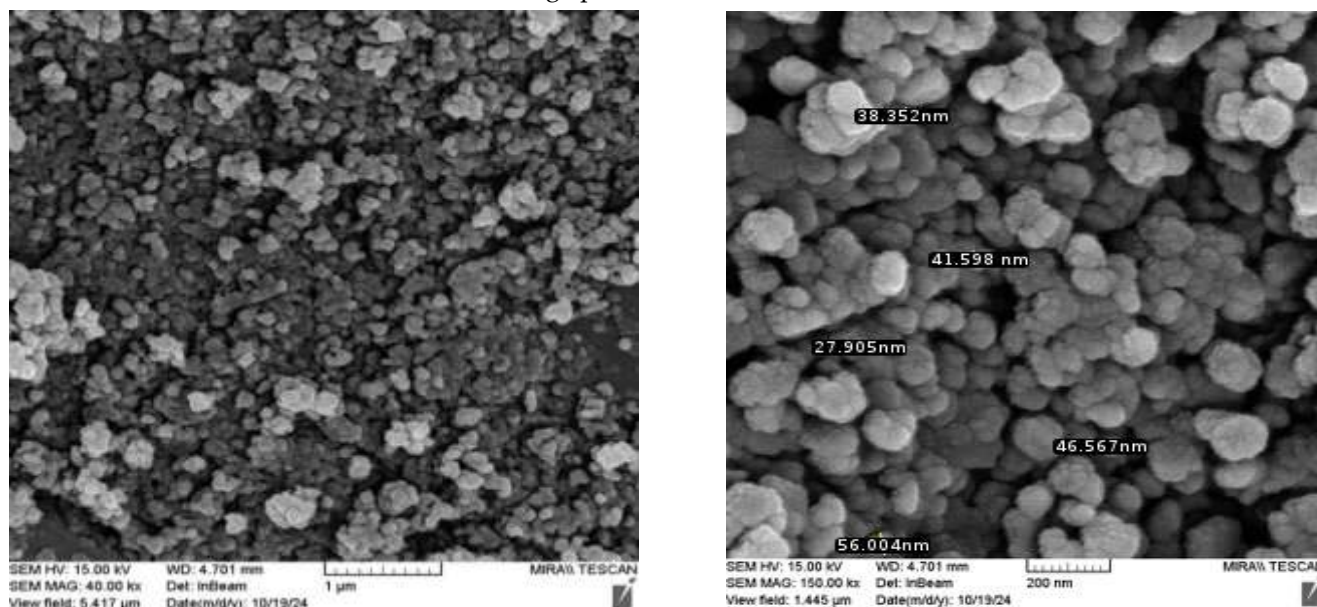


Figure 4. FESEM of ZnO-NPs synthesized by *L. rhamnosus* at two magnification power 15000kx and 4000 kx

## 3 -X-Ray Diffraction (XRD) Profiling

The crystalline characteristics of the ZnO-NPs biosynthesized using *L. rhamnosus* were examined by X-ray diffraction (XRD) [28]. The crystalline size (D) was calculated using the Scherrer equation:

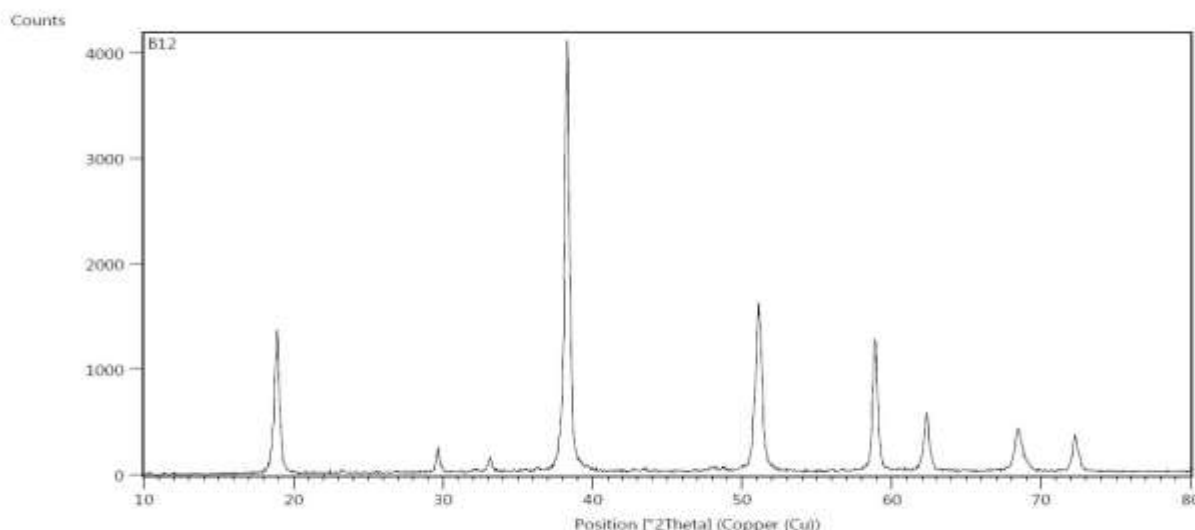
$$D = \frac{K\lambda}{\beta \cos \theta}$$

K is the shape factor (0.9)

$\lambda$  is the wavelength of Cu K $\alpha$  radiation (0.15406 nm)

$\beta$  is the full width at half maximum (FWHM) of the prominent peak in radians  $\theta$  is the Bragg diffraction angle corresponding to half of the reported  $2\theta$  value [29]. Based on the experimental data, the most prominent diffraction peak was recorded at  $2\theta = 38.275$  with FWHM 0.006632 rad [30].

So the average crystalline size was 22.13 nm for the biosynthesized ZnO by *L. rhamnosus*, as shown in **Figure 5**.



**Figure 5.** XRD analysis of biogenic ZnO nanoparticle biosynthesis from *L. rhamnosus*

#### 4 -Fourier Transform Infrared Spectroscopy (FTIR)

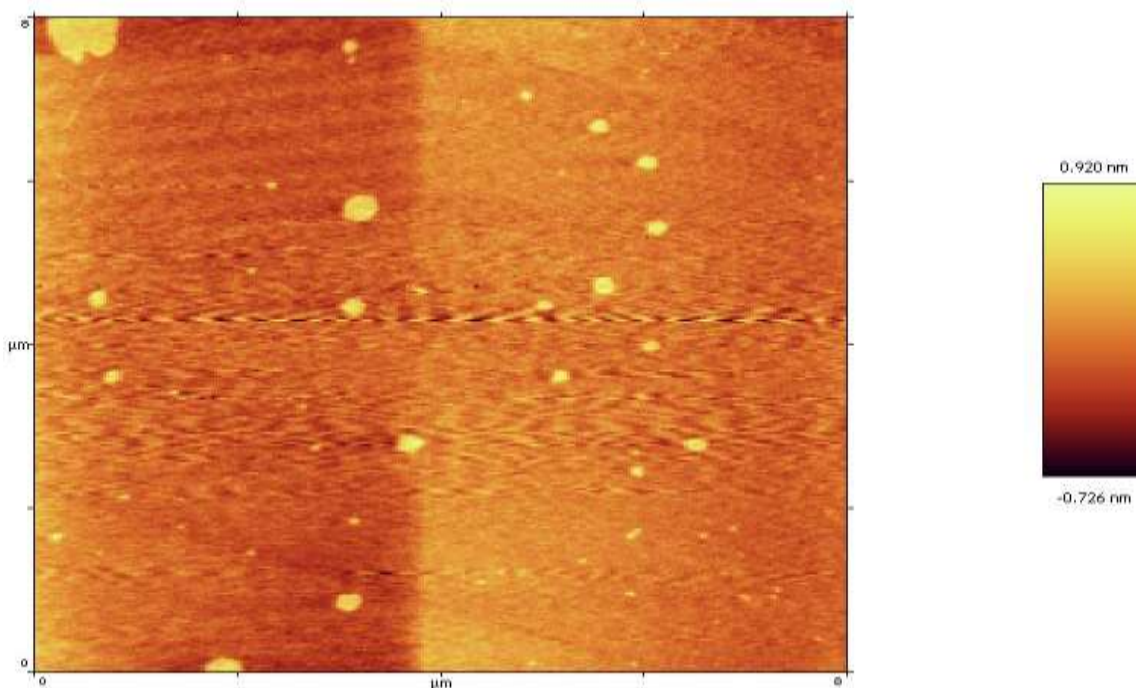
FTIR spectroscopy was performed to identify the functional groups instrumental in the biogenic synthesis and surface stabilization of ZnO nanoparticles (ZnO-NPs) (Figure 6) [31]. The pronounced absorption peak at  $563\text{ cm}^{-1}$  is attributed to Zn–O bond stretching, unequivocally verifying ZnO-NP formation [32]. Amide II modes and carboxylate ( $\text{COO}^-$ ) stretching are attributed to spectral characteristics seen between  $1448$  and  $1580\text{ cm}^{-1}$ , indicating the functional significance of bacterial proteins in capping the nanostructures [33]. Furthermore, coupled O–H and N–H stretching vibrations of adsorbed water molecules, hydroxyl residues, and surface-bound proteins produce the broad absorption band spanning  $3200$ – $3300\text{ cm}^{-1}$ , whereas aliphatic C–H stretching is reflected in the small peak at  $2920\text{ cm}^{-1}$ .

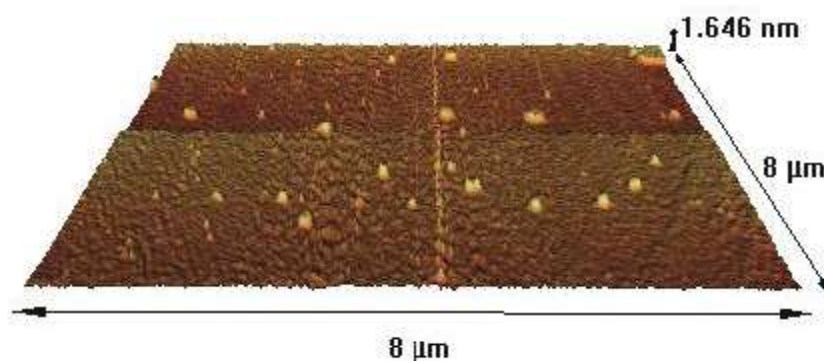


**Figure 6.** FTIR analysis of biogenic ZnO nanoparticles mediated by *L.rhamnosus*

### 5 -Atomic Force Microscopy (AFM) Topography

Using 3D Atomic Force Microscopy, the surface topography and nanoscale roughness of the ZnO-NPs produced by *L. rhamnosus* were evaluated (Figure 7). The vertical height values obtained from topographic measurements ranged from  $-0.726$  to  $0.920$  nm, meaning that the peak-to-valley height difference was  $1.646$  nm [34]. Because bacterial metabolites actively participate in passivating the surface and controlling particle development, this tiny surface irregularity indicates fine nanoscale roughness.



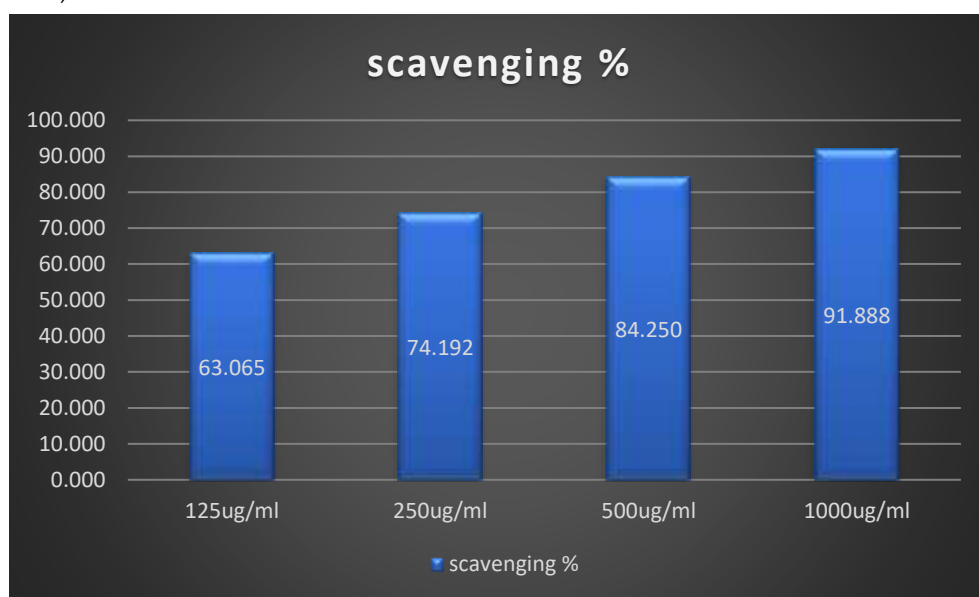


**Figure 7.** AFM analysis of biogenic ZnO nanoparticles mediated by *L.rhamnosus*

### Biological Evaluation of Biogenic ZnO-NPs

#### 1 -In Vitro Antioxidant and Radical Scavenging Efficacy

The produced ZnO-NPs demonstrated a definite concentration-dependent antioxidant activity, with the DPPH radical scavenging efficiency gradually increasing with nanoparticle concentration. Figure 8 illustrates that at concentrations of 500, 250, and 125  $\mu\text{g/mL}$ , free radical inhibition was highest at 1000  $\mu\text{g/mL}$  (91.888%), followed by 84.250%, 74.192%, and 63.065%.



**Figure 8.** Antioxidant activity of ZnO NPs

#### In Vitro Hemolytic Safety Profile

The safety profile and blood compatibility of the biosynthesized zinc oxide nanoparticles (ZnO-NPs) made with the *L. rhamnosus* strain were assessed by measuring their hemolytic activity. As presented in Table 1, the calculated hemolysis was 1.60% at 1000  $\mu\text{g/mL}$ . At concentrations of 125, 250, and 500  $\mu\text{g/mL}$ , the absorbance values were lower than those of the PBS negative control; therefore, the corresponding negative calculated values were considered 0% after baseline correction. Under the tested in vitro conditions, the ZnO-NPs showed low hemolytic activity [35].

**Table 1.** Hemolysis activity of ZnO NPs biosynthesis from *L.rhamnosus*

| concentration (ug/ml) | test result | hemolysis % |
|-----------------------|-------------|-------------|
| 1000                  | 0.4046      | 1.6         |
| 500                   | 0.3584      | 0           |
| 250                   | 0.3554      | 0           |
| 125                   | 0.3441      | 0           |
| POSITIVE              | 1.924       | 100         |
| NEGATIVE              | 0.3799      | 0           |

## Discussion

### Selection and identification of Biosynthetic Probiotic Isolates

The differences observed among bacterial isolates in nanoparticle production efficiency can be attributed to variations in metabolic activity, enzyme secretion, and the composition of extracellular biomolecules [36]. Microbial-mediated nanoparticle synthesis is highly dependent on species and strain specific characteristics as different microorganisms possess distinct biochemical pathways responsible for metal ion transformation, nanoparticle nucleation and stabilization [37]. During extracellular biosynthesis, bacterial cells contribute to the formation of nanoparticles by releasing reducing agents such as enzymes, proteins, and other metabolites. These biomolecules catalyze the conversion of metal ions into stable nanostructures and may then function as natural capping agents for particle growth and nanoparticle stability [38]. Thus, the production ability of ZnO-NPs demonstrated by the *L. rhamnosus* isolate underlines the importance of strain selection in microbial nanofabrication and supports the potential use of probiotic bacteria as eco-friendly platforms for the synthesis of ZnO nanoparticles [39].

### Physicochemical Characterization of Biosynthesized ZnO-NPs

This intense UV absorption peak around 320-350 nm is due to the intrinsic bandgap transition of electrons from the valence band to the conduction band ( $O_{2p} \rightarrow Zn_{3d}$ ).

The absorption band observed at approximately 320–350 nm  was shifted toward shorter wavelengths compared with the typical absorption edge of bulk ZnO at approximately 370–380 nm. This shift may be associated with the nanoscale dimensions of the synthesized particles [40]. However, confirmation of changes in the optical band gap would require additional analysis, such as a Tauc plot. The relatively low absorption in the visible region indicates that the synthesized ZnO-NPs had limited visible-light absorption under the tested conditions [41].

The aggregation observed in FE-SEM micrographs arises from the high surface energy of nanoparticles due to their high surface-to-volume ratio, leading to strong interparticle interactions and attractive forces like van der Waals forces.

The measured nanoscale dimensions show that the biological synthesis process was successful in forming ZnO nanoparticles [42], [43]. The presence of bacterial biomolecules, such as proteins, enzymes, and extracellular metabolites, which can function as reducing and capping agents to regulate nucleation and crystal growth during nanoparticle formation, may be linked to the comparatively uniform particle size distribution (average 41.5 nm). The current study's morphology and size parameters are similar to those found

in earlier studies on the microbial synthesis of ZnO nanoparticles found that ZnO nanoparticles produced using *Lactobacillus plantarum* had spherical morphology and nanoscale dimensions, demonstrating the capacity of *Lactobacillus* species to promote ZnO nanoparticle formation through biological processes. Similarly, electron imaging revealed that ZnO nanoparticles produced with *Lactobacillus* species had a spherical shape and an average size of around 32 nm, along with the development of nanoparticle aggregates changes in bacterial species, precursor content, pH, incubation conditions, and the makeup of extracellular biomolecules involved in nanoparticle formation can all contribute to changes in particle size between studies [44].

The XRD pattern indicated the crystalline nature of the biosynthesized ZnO-NPs. The calculated crystallite size of 22.13 nm was smaller than the average particle size observed by FESEM (41.5 nm), which may reflect the aggregation of smaller crystallites into larger particles. However, the possible involvement of biomolecules derived from the *L. rhamnosus* cell-free supernatant in nanoparticle stabilization is better supported by the FTIR findings than by the XRD pattern [45].

The FTIR band at  $563\text{ cm}^{-1}$  unequivocally verifies Zn–O bond formation. Amide II and carboxylate ( $\text{COO}^-$ ) bands ( $1448\text{--}1580\text{ cm}^{-1}$ ) signify the functional role of bacterial proteins in capping the nanostructures. These results support the literature on *Lactobacillus*-assisted nanoparticle manufacturing by confirming that biomolecules generated from *L. rhamnosus* performed dual roles as reducing and capping agents [46].

The vertical height values obtained from AFM measurements ranged from  $-0.726$  to  $0.920\text{ nm}$ , indicating a  $1.646\text{ nm}$  peak-to-valley height difference. Bacterial metabolites linked to the stabilization and development of nanoparticle surfaces may have an impact on this surface topography pointed out that biomolecular interactions with metal precursors directly affect the final surface architecture, while highlighted AFM's capacity to resolve surface characteristics and spatial arrangement in green nano-synthesis. When taken as a whole, these characteristics demonstrate that *L. rhamnosus* is an efficient stabilizing agent that produces environmentally benign ZnO-NPs with regulated surface characteristics suitable for biological applications [47].

### Biological Evaluation of Biogenic ZnO-NPs

The concentration-dependent enhancement in DPPH radical scavenging capability may be attributed to the greater availability of active nanoparticle surfaces and the enhanced interaction between ZnO-NPs and free radicals. Similar concentration-dependent antioxidant responses have been reported for green-synthesized ZnO nanoparticles, where increasing nanoparticle concentration resulted in higher DPPH radical inhibition [48]. The DPPH assay is based on the ability of antioxidant compounds to donate electrons or hydrogen atoms to the stable DPPH radical, causing the reduction of the violet-colored DPPH solution into a yellow-colored non-radical form [49]. The antioxidant activity of ZnO nanoparticles has been associated with their nanoscale surface properties and the presence of biologically derived compounds that can enhance their interaction with reactive molecules, overall, the results indicate that *L. rhamnosus*-mediated ZnO-NPs possess considerable antioxidant potential and may have promising applications in biological and biomedical fields [50].

The low hemolytic activity observed at the tested concentrations, including  $1.60\%$  hemolysis at  $1000\text{ }\mu\text{g/mL}$ , may be associated with the biological surface modification of ZnO nanoparticles during microbial synthesis. Biomolecules derived from *L. rhamnosus*, such as proteins and other extracellular compounds, may interact with the nanoparticle surface and reduce direct contact between ZnO and erythrocyte membranes. Similar studies have demonstrated that green-synthesized ZnO nanoparticles can exhibit favorable hemocompatibility profiles, with low hemolytic effects depending on nanoparticle concentration and surface characteristics [51].

#### 4. Conclusion

Probiotic-mediated biosynthesis using *Lactocaseibacillus rhamnosus* represents a simple, eco-friendly, and effective approach for producing ZnO nanoparticles; the characterization technique confirmed the formation of ZnO at nanoscale features. The biological evaluation showed ZnO-NPs exhibited concentration-dependent antioxidant activity, suggesting their ability to effectively scavenge free radicals. Finally, the nanoparticles showed low hemolytic activity under the tested in vitro conditions, with 1.60% hemolysis at the highest concentration of 1000 µg/mL.

#### Conflict of interest

The authors declare that they have no competing interests.

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