

Biocompatible nanobiohybrid of maghemite nanoparticle-coated probiotics: A synergistic platform for enhanced antibacterial, antibiofilm, and antioxidant activities

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ABSTRACT

The rise in free radical species and bacterial infections accelerates biofilm formation, impeding wound healing and leading to severe complications, thereby posing a significant global threat to public health. In this study, we synthesized a novel nanobiohybrid material by coating maghemite nanoparticles (γ -Fe₂O₃ NPs) onto the surface of *Lactobacillus rhamnosus* (LR) probiotics to address bacterial infections, inhibit biofilm formation, and scavenge free radicals. The γ -Fe₂O₃ NPs–LR nanobiohybrid was characterized using PXRD, FTIR, XPS, TEM, FESEM, zeta potential analysis, and UV–Vis spectroscopy, confirming the successful interaction between the γ -Fe₂O₃ NPs and LR. The antibacterial activity of the nanobiohybrid at 1000 μ g mL^{−1} was assessed by measuring the zone of inhibition against *L. monocytogenes* (15.17 $\pm$ 0.29 mm), *S. succinus* (12.83 $\pm$ 0.29 mm), *S. thompson* (5.33 $\pm$ 0.29 mm), and *E. coli* O157:H7 (7.67 $\pm$ 0.29 mm), revealing significantly higher inhibitory effects compared to γ -Fe₂O₃ NPs and LR alone. Antibiofilm assays demonstrated the nanobiohybrid's superior ability to inhibit and disrupt established biofilms. Additionally, the antioxidant potential was evaluated using the DPPH scavenging assay, which showed enhanced scavenging activity (66.57 $\pm$ 0.53% at 500 μ g mL^{−1}). Importantly, cytotoxicity studies on mouse embryonic fibroblast (MEF) and human intestinal (Caco-2) cell lines confirmed that the nanobiohybrid exhibits excellent biocompatibility at relevant concentrations. This study demonstrates that the γ -Fe₂O₃ NPs–LR nanobiohybrid is a promising biocompatible synergistic platform for diverse biomedical applications, including infection control, wound healing, and oxidative stress management.

1. Introduction

The significance of probiotic microorganisms in human health has garnered increasing attention due to their health-promoting effects, particularly in gut health, immune modulation, and protection against microbial infections [1]. Among them, *Lactobacillus rhamnosus* (LR) is a well-established probiotic known for its antibacterial, antibiofilm, and antioxidant properties, primarily attributed to the production of lactic acid, bacteriocins, and hydrogen peroxide [2–4]. Its strong gastrointestinal resilience and ability to regulate oxidative stress further support its therapeutic relevance [5,6].

Building on the beneficial properties of probiotics such as LR, the integration of nanomaterials offers opportunities to enhance their functionality. Among various nanomaterials, maghemite nanoparticles

(γ -Fe₂O₃ NPs) have attracted interest due to their high surface area, redox activity, and ability to generate reactive oxygen species (ROS), contributing to antibacterial, antibiofilm, and antioxidant applications [7–9]. Their chemical stability and favorable surface charge make them suitable candidates for biomedical applications. Notably, γ -Fe₂O₃ NPs were selected instead of noble metal NPs such as gold, silver, or platinum because of their cost-effectiveness, earth abundance, and ease of large-scale preparation. However, concerns regarding cytotoxicity and environmental safety limit their direct biomedical use, necessitating biologically compatible alternatives.

To address these limitations, integrating nanomaterials with probiotic systems has emerged as a strategy to enhance antibacterial efficacy while improving biocompatibility. In such nanobiohybrid systems, NPs contribute antibacterial and ROS-mediated activities, while

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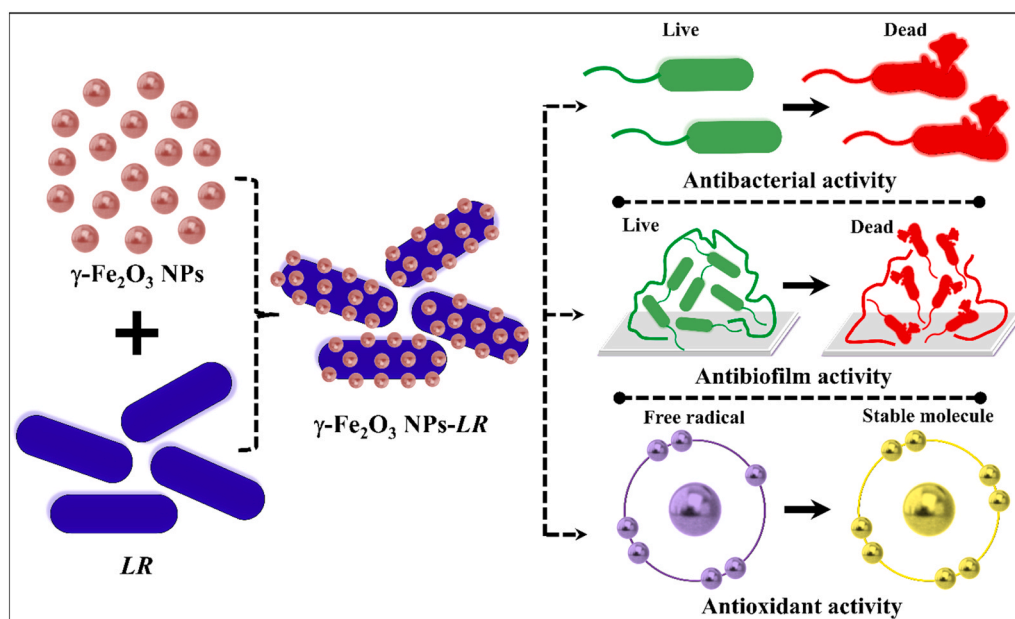
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Scheme 1. Schematic illustration of the synthesis of γ -Fe₂O₃ NPs-LR nanobiohybrid and its demonstrated antibacterial, antibiofilm, and antioxidant activities.

probiotics support biological safety and sustained antibacterial and antibiofilm effects through the secretion of bioactive metabolites [10]. LR exhibits inherent tolerance toward NP exposure, making it a suitable biological scaffold for NP integration.

The synthesis strategy plays a crucial role in preserving probiotic viability and achieving functional synergy. Electrostatic interaction provides a mild, aqueous, and scalable approach that enables uniform NP attachment without compromising bacterial morphology or activity [11]. Encapsulation and direct synthesis methods are often complex and may involve harsh conditions [12], whereas the electrostatic interaction method allows interfacial tuning while maintaining cell viability and morphology. Although several probiotic-based nanocomposites have been reported for antibacterial or antibiofilm applications [13–15], electrostatically assembled γ -Fe₂O₃ NPs-LR nanobiohybrid has not been explored for simultaneous antibacterial, antibiofilm, and antioxidant enhancement.

In this study, γ -Fe₂O₃ NPs-LR nanobiohybrid was prepared via electrostatic self-assembly and evaluated for biomedical applications (Scheme 1). The physicochemical characteristics were analyzed using various techniques. The hybrid exhibited enhanced antibacterial, antibiofilm, and antioxidant activities compared to its individual components. This study demonstrates that combining γ -Fe₂O₃ NPs with LR offers a feasible approach for multifunctional applications. To the best of our knowledge, this is the first report on the use of γ -Fe₂O₃ NPs-LR nanobiohybrid for multifunctional applications.

2. Materials and methods

2.1. Chemicals and microorganisms

Hydrochloric acid (HCl), sodium nitrate (NaNO₃), ferric chloride hexahydrate (FeCl₃•6 H₂O), ferrous chloride tetrahydrate (FeCl₂•4 H₂O), sodium hydroxide (NaOH), perchloric acid (HClO₄), bile salt, De Man Rogosa and Sharpe (MRS) broth, Mueller-Hinton broth (MHB), Mueller-Hinton agar (MHA), phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Doxorubicin hydrochloride (DOX), WST-8 dye, ascorbic acid, crystal violet dye, Ampicillin, and Ciprofloxacin were purchased from Sigma-Aldrich (USA). The LIVE/DEAD™ BacLight™ Bacterial Viability Kit (L7012) was obtained from Invitrogen, Thermo Fisher Scientific (USA),

and 2,2-diphenyl-1-picrylhydrazyl (DPPH) was sourced from BIOZOA Biological Supply (South Korea). Penicillin-streptomycin was acquired from Gibco (USA). *Lactobacillus rhamnosus* (KCTC 5033) was procured from the Korean Collection for Type Cultures (KCTC), while *Salmonella thompson* (Wild-type 4021) was provided by the Korean Ministry of Food and Drug Safety (MFDS, South Korea). *Listeria monocytogenes* (ATCC 15313), *Staphylococcus succinus* (ATCC 15802), and *Escherichia coli* O157:H7 (ATCC 43894) were obtained from the American Type Culture Collection (ATCC). Caco-2 and mouse embryonic fibroblast (MEF) cell lines were sourced from the National Centre for Cell Science (NCCS, India).

2.2. Synthesis of γ -Fe₂O₃ NPs

γ -Fe₂O₃ NPs were synthesized via a co-precipitation method followed by oxidation using Massart's protocol [16]. A cationic solution (Solution A) was prepared by dissolving 0.050 M FeCl₃•6 H₂O and 0.025 M FeCl₂•4 H₂O in deionized water. An anionic solution (Solution B) was prepared by mixing 1 M NaOH with 1 M NaNO₃. Solution B was gradually added to Solution A under vigorous stirring in a three-necked flask at ambient temperature, while maintaining the pH at 11.0 ± 0.1. The resulting magnetite was oxidized to γ -Fe₂O₃ using 1 M HClO₄ and aged for 30 min. The product was collected by centrifugation at 4000 rpm and thoroughly washed with deionized water to eliminate residual impurities. The purified NPs were dried under vacuum at 40 °C. All procedures were conducted under a nitrogen atmosphere to prevent exposure to environmental contaminants.

2.3. Cultivation of LR

LR was cultured in MRS broth under anaerobic conditions at 37 °C overnight and preserved at –80 °C in MRS broth supplemented with 25% glycerol.

2.4. Synthesis of γ -Fe₂O₃ NPs-LR nanobiohybrid

The synthesized γ -Fe₂O₃ NPs were dispersed in PBS at a concentration of 500 µg mL^{–1}. Equal volumes (500 µL) of the γ -Fe₂O₃ NP dispersion and the LR PBS suspension (10⁸ CFU mL^{–1}) were mixed and incubated at 37 °C with shaking for 1 h. The resulting γ -Fe₂O₃ NPs-LR

nanobiohybrid was separated by centrifugation at 4000 rpm for 10 min and subsequently freeze-dried.

2.5. Material characterization

Details of the characterization techniques are provided in [Supporting Information S1](#).

2.6. Effect of γ -Fe₂O₃ NPs coating on LR

2.6.1. Plate count method

To evaluate the effect of γ -Fe₂O₃ NPs on LR viability, both LR and γ -Fe₂O₃ NPs–LR samples were assessed using the plate count method. Each sample was serially diluted in PBS, and 100 μ L of the appropriate dilutions were plated onto MRS agar. The plates were incubated at 37 °C for 24 h. After incubation, colony-forming units were counted on plates containing 15–300 distinguishable colonies.

2.6.2. Confocal laser scanning microscopy (CLSM)

The viability of LR in the presence and absence of γ -Fe₂O₃ NPs was further evaluated using CLSM in conjunction with the LIVE/DEAD™ BacLight™ Bacterial Viability Kit. This assay employs two nucleic acid-binding fluorescent dyes: SYTO-9, which stains live cells, and propidium iodide (PI), which penetrates membrane-compromised or dead cells. Suspensions of LR and γ -Fe₂O₃ NPs–LR were prepared and stained according to the manufacturer's instructions. A staining solution consisting of equal volumes of SYTO-9 and PI was prepared, and 3 μ L of this solution was added to 1000 μ L of each bacterial suspension. Samples were mixed thoroughly and incubated in the dark at room temperature for 15 min. Subsequently, 5 μ L of each stained sample was mounted onto a microscope slide and covered with a coverslip. Fluorescence imaging was performed using CLSM, with excitation wavelengths of 488 nm for SYTO-9 and 555 nm for PI.

2.6.3. Growth curve

To investigate the effect of γ -Fe₂O₃ NPs on LR activity, the bacterial growth profile was assessed *in vitro* with and without γ -Fe₂O₃ NPs treatment. Fresh cultures of LR and the γ -Fe₂O₃ NPs–LR nanobiohybrid were adjusted in MRS broth to an initial optical density (OD) of 0.05 at 600 nm. A volume of 200 μ L from each culture was transferred into the wells of a 96-well plate and incubated at 37 °C for 48 h. Bacterial proliferation was monitored by measuring turbidity at regular intervals using a microplate reader. Growth kinetics were evaluated based on changes in OD₆₀₀ values over the incubation period.

2.6.4. Bile salt tolerance

The tolerance of LR and the γ -Fe₂O₃ NPs–LR nanobiohybrid to bile salts was assessed using a microplate-based assay. MRS broth was supplemented with bile salts at concentrations of 0.3 and 0.5%, while unsupplemented broth (0%) served as the control. A total of 20 μ L of each bacterial culture was added to 180 μ L of the respective bile salt-modified medium in a 96-well plate. Cultures were incubated at 37 °C for 12 h, and bacterial growth was monitored hourly by measuring OD₆₀₀ using a microplate reader.

2.6.5. Acid tolerance

Acid tolerance of LR and the γ -Fe₂O₃ NPs–LR nanobiohybrid was evaluated under different pH conditions. MRS broth was adjusted to pH 2.0 and 3.0 using 1 M HCl, with pH 6.0 serving as the control. Each well received 180 μ L of the pH-adjusted medium and 20 μ L of bacterial suspension. The plate was incubated at 37 °C for 12 h, and OD₆₀₀ measurements were recorded hourly to monitor bacterial proliferation under acidic stress.

2.7. Antibacterial activity

2.7.1. Agar disc diffusion assay

To evaluate the antibacterial activity of γ -Fe₂O₃ NPs, LR, and the γ -Fe₂O₃ NPs–LR nanobiohybrid, disc diffusion assay was performed against Gram-positive (*L. monocytogenes* and *S. succinus*) and Gram-negative (*S. thompson* and *E. coli* O157:H7) bacterial strains. All strains were cultured to match the 0.5 McFarland turbidity standard (1.5×10^8 CFU mL⁻¹), and 100 μ L of each suspension was evenly spread onto MHA plates. Test solutions were prepared in sterile distilled water, and 30 μ L of each concentration (250, 500, and 1000 μ g mL⁻¹) was applied to sterile 6 mm blank discs. Plates were incubated at 37 °C overnight, and zones of inhibition (ZOI) were measured to assess antibacterial performance. Ampicillin and Ciprofloxacin (10 μ g disc⁻¹) served as positive controls, while discs loaded with sterile distilled water acted as negative controls [17].

2.7.2. Broth dilution method

To evaluate the antibacterial efficacy of γ -Fe₂O₃ NPs, LR, and the γ -Fe₂O₃ NPs–LR nanobiohybrid, the minimum inhibitory concentration (MIC) was determined using the broth macrodilution method. Two-fold serial dilutions of the samples were prepared in MHB within a 96-well microtiter plate at concentrations ranging from 500 to 0.97 μ g mL⁻¹. Subsequently, 100 μ L of a bacterial suspension (*L. monocytogenes*, *S. succinus*, *S. thompson*, and *E. coli* O157:H7), adjusted to 1.5×10^8 CFU mL⁻¹ was added to each well. The plates were then incubated overnight at 37 °C. Bacterial growth was assessed using a microplate reader. Wells containing only bacterial cultures served as positive controls, while culture-free medium served as the negative control. To determine the minimum bactericidal concentration (MBC), aliquots from non-turbid wells, including those at the MIC level, were plated on MHA and incubated overnight at 37 °C.

2.8. Antibiofilm activity

2.8.1. Inhibition of biofilm formation

To assess the inhibitory effect of γ -Fe₂O₃ NPs, LR, and the γ -Fe₂O₃ NPs–LR nanobiohybrid on biofilm formation, a crystal violet assay was conducted using a 96-well microtiter plate. A 100 μ L aliquot of bacterial culture (1×10^6 CFU mL⁻¹) of *L. monocytogenes*, *S. succinus*, *S. thompson*, and *E. coli* O157:H7 was added to each well, followed by 100 μ L of the test samples at concentrations of 250, 500, and 1000 μ g mL⁻¹. The plates were incubated statically at 37 °C overnight. After incubation, non-adherent cells were removed by washing each well three times with PBS. To stain the attached biofilm, 200 μ L of 0.1% crystal violet solution was added to each well and incubated at room temperature for 15 min. Excess dye was removed by washing the wells three times with PBS, after which the stained biofilm was solubilized using 200 μ L of 96% ethanol. The plates were then incubated at 37 °C for an additional 15 min. OD was measured at 570 nm using a microplate reader. Untreated bacterial cultures served as controls. Biofilm inhibition (%) was calculated according to Eq. (1) [18]:

$$\text{Biofilm inhibition(\%)} = \frac{\text{OD}_{\text{untreated}} - \text{OD}_{\text{treated}}}{\text{OD}_{\text{untreated}}} \times 100 \quad (1)$$

2.8.2. Eradication of preformed biofilm

A 96-well microtiter plate was employed to evaluate the eradication of preformed biofilms. Bacterial strains (*L. monocytogenes*, *S. succinus*, *S. thompson*, and *E. coli* O157:H7) were cultured in the wells and incubated statically at 37 °C overnight to facilitate biofilm formation. Following incubation, the medium was carefully removed, and each well was gently rinsed with PBS to eliminate non-adherent cells. Subsequently, 100 μ L of fresh medium and 100 μ L of γ -Fe₂O₃ NPs, LR, or γ -Fe₂O₃ NPs–LR at concentrations of 250, 500, and 1000 μ g mL⁻¹ were added to each well. The plates were then incubated for an additional

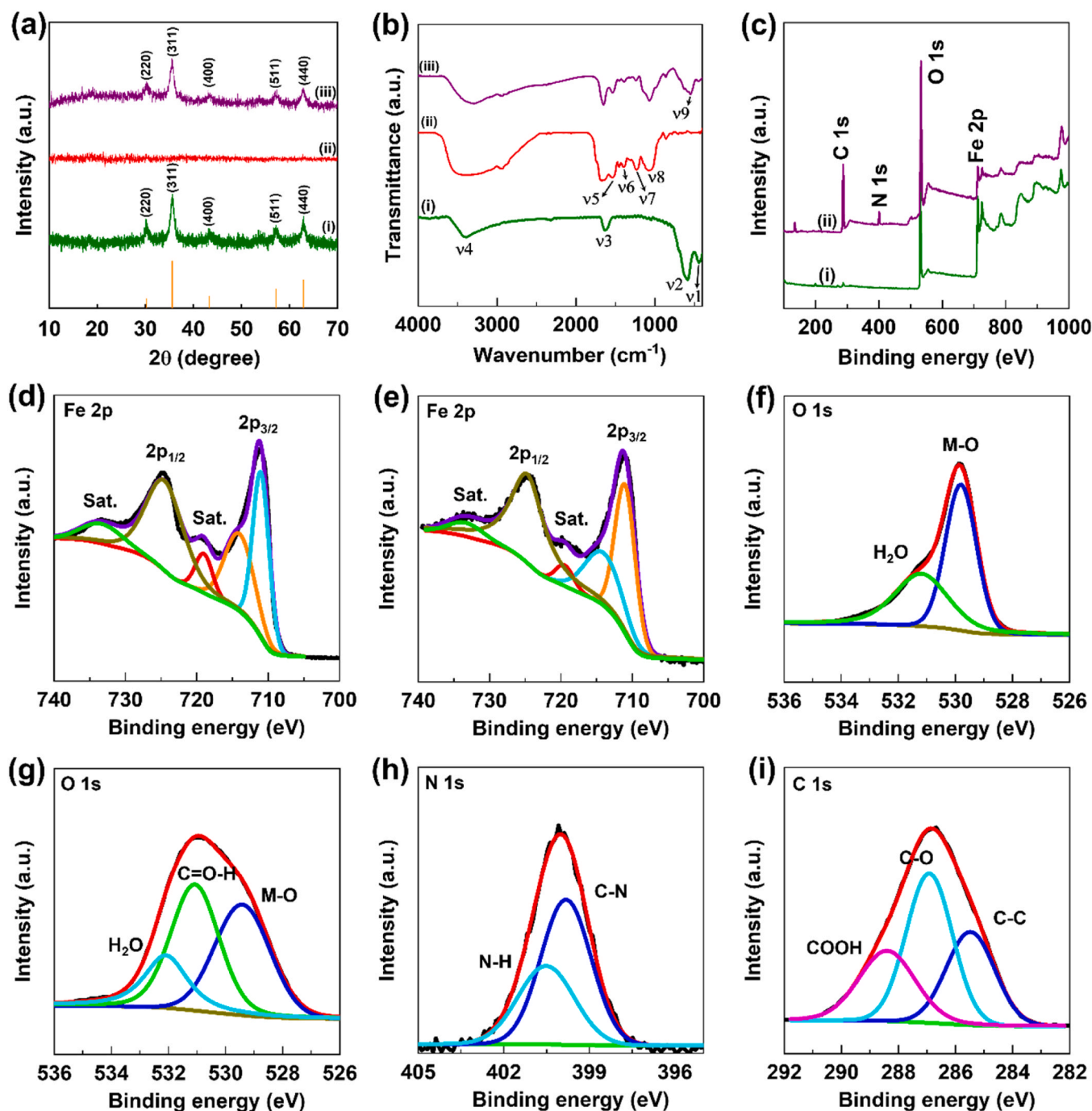


Fig. 1. (a) PXRD patterns of (i) γ -Fe₂O₃ NPs, (ii) LR, and (iii) γ -Fe₂O₃ NPs–LR nanobiohybrid, and the reference pattern of γ -Fe₂O₃ (orange; JCPDS no. 39–1346). (b) FTIR spectra of (i) γ -Fe₂O₃ NPs, (ii) LR, and (iii) γ -Fe₂O₃ NPs–LR nanobiohybrid. XPS spectra: (c) Survey spectra of (i) γ -Fe₂O₃ NPs and (ii) γ -Fe₂O₃ NPs–LR; high-resolution Fe 2p spectra of (d) γ -Fe₂O₃ NPs and (e) γ -Fe₂O₃ NPs–LR, O 1s spectra of (f) γ -Fe₂O₃ NPs and (g) γ -Fe₂O₃ NPs–LR, (h) N 1s, and (i) C 1s spectra of γ -Fe₂O₃ NPs–LR.

12 h at 37 °C. After the treatment period, wells were rinsed again with PBS to remove planktonic cells. Residual biofilms were stained with 200 μ L of 0.1% crystal violet solution for 15 min at room temperature. Excess dye was removed by washing the wells three times with PBS, and the stained biofilm was solubilized with 200 μ L of 96% ethanol. The OD was measured at 570 nm using a microplate reader. Untreated wells served as controls. Biofilm eradication (%) was calculated using Eq. (2):

$$\text{Biofilm eradication(\%)} = \frac{\text{OD}_{\text{untreated}} - \text{OD}_{\text{treated}}}{\text{OD}_{\text{untreated}}} \times 100 \quad (2)$$

2.9. Antioxidant activity

The antioxidant activity of γ -Fe₂O₃ NPs, LR, and the γ -Fe₂O₃ NPs–LR nanobiohybrid was evaluated using the DPPH assay, following the protocol described by Liu et al. [3]. Test samples were prepared at concentrations ranging from 100 to 500 μ g mL⁻¹. In a 96-well microtiter plate, 180 μ L of 0.1 mM DPPH solution was mixed with 20 μ L of each sample. The plate was covered with aluminum foil and incubated at room temperature for 30 min. Ascorbic acid and untreated DPPH solution served as the positive and negative controls, respectively. The OD was measured at 517 nm using a microplate reader. The percentage of

free radical scavenging activity (FRSA) was calculated according to Eq. (3):

$$\text{FRSA}(\%) = \frac{\text{OD}_{\text{untreated}} - \text{OD}_{\text{treated}}}{\text{OD}_{\text{untreated}}} \times 100 \quad (3)$$

2.10. Biocompatibility assay

The biocompatibility of $\gamma\text{-Fe}_2\text{O}_3$ NPs and the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid was assessed in MEF and Caco-2 cells using the WST-8 assay. Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin, and incubated at 37 °C under 5% CO_2 and 95% humidity. Subsequently, cells were seeded into 96-well plates at a density of 5×10^3 cells per well and incubated for 24 h. Following incubation, cells were treated with varying concentrations ($0\text{--}500 \mu\text{g mL}^{-1}$) of $\gamma\text{-Fe}_2\text{O}_3$ NPs or $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR and further incubated for 24 h. Then, 10 μL of WST-8 solution (10% v/v) was added to each well and incubated for 1 h. Cell viability was determined by measuring the OD at 450 nm using a microplate reader. DOX served as the positive control, while untreated cells maintained in DMEM acted as the negative control [19]. Cell viability (%) was calculated using Eq. (4):

$$\text{Cell viability}(\%) = \frac{\text{OD}_{\text{treated}}}{\text{OD}_{\text{untreated}}} \times 100 \quad (4)$$

2.11. Statistical analysis

All experiments were conducted in triplicate, and results are reported as mean values $\pm$ standard deviation (SD).

3. Results and discussion

3.1. Physicochemical characterization of the $\gamma\text{-Fe}_2\text{O}_3$ NPs, LR, and $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid

Powder X-ray diffraction (PXRD) analysis was conducted to investigate the crystal structures of the synthesized $\gamma\text{-Fe}_2\text{O}_3$ NPs, LR, and the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid (Fig. 1a). The PXRD pattern of the $\gamma\text{-Fe}_2\text{O}_3$ NPs displayed distinct diffraction peaks at 30.2° , 35.5° , 43.3° , 57.2° , and 62.7° , corresponding to the (220), (311), (400), (511), and (440) crystal planes, respectively [20]. These reflections are characteristic of the $\gamma\text{-Fe}_2\text{O}_3$ phase and align well with the standard JCPDS card no. 39–1346, confirming the successful synthesis of $\gamma\text{-Fe}_2\text{O}_3$ NPs. The average crystallite size of the $\gamma\text{-Fe}_2\text{O}_3$ NPs was 8.9 ± 0.8 nm. The crystallite size calculated using the Scherrer equation is in close agreement with previously reported iron oxide NPs [21,22]. In addition, the observed diffraction peak broadening reveals the nanoscale nature of the $\gamma\text{-Fe}_2\text{O}_3$ NPs. In contrast, the PXRD pattern of LR showed a broad and featureless profile. This indicates its amorphous nature, which is typical of biological materials and reflects their disordered molecular arrangement. The PXRD pattern of the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid retained the characteristic peaks of $\gamma\text{-Fe}_2\text{O}_3$, suggesting that incorporation of LR did not significantly disrupt the crystalline structure of the $\gamma\text{-Fe}_2\text{O}_3$ NPs. However, a marked reduction in peak intensity was observed, implying possible interactions between the NPs and LR, which may influence crystallinity or promote homogeneous dispersion of $\gamma\text{-Fe}_2\text{O}_3$ within the hybrid matrix. Overall, the PXRD results confirm the successful formation of the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid and provide essential insight into the structural integrity and phase retention of the inorganic component within the composite.

The nature of chemical bonding in the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid was investigated using Fourier-transform infrared spectroscopy (FTIR), as shown in Fig. 1b. The FTIR spectrum of pristine $\gamma\text{-Fe}_2\text{O}_3$ NPs exhibits two characteristic absorption bands at 435 cm^{-1} (ν_1) and 627 cm^{-1} (ν_2), corresponding to Fe–O vibrational modes [23,24]. Additional bands at 1625 cm^{-1} (ν_3) and 3404 cm^{-1} (ν_4) are attributed to the bending vibration of molecular water and the stretching vibration of

surface hydroxyl (–OH) groups, respectively [25,26]. These surface –OH groups are essential for hydrogen bonding with functional moieties on the LR cell surface, such as carboxyl and amine groups. In the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid, a noticeable shift and broadening of the –OH stretching vibration to 3300 cm^{-1} suggests the formation of strong hydrogen bonds between $\gamma\text{-Fe}_2\text{O}_3$ NPs and LR. The $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid commonly displays a peak at 1653 cm^{-1} , which is assigned to overlapping contributions from water bending and the amide I band [27]. Furthermore, the FTIR spectra of both LR and the nanobiohybrid display distinct absorption peaks at 1533 cm^{-1} (ν_5), 1399 cm^{-1} (ν_6), 1240 cm^{-1} (ν_7), and 1073 cm^{-1} (ν_8), which correspond to amide bonds, symmetric COO^- stretching, and CH_2 bending of fatty acid, and asymmetric PO_2^- stretching from DNA, respectively [28–32]. Notably, the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid also shows intensified and strong Fe–O absorption band at 557 cm^{-1} (ν_9) [33]. This shifts in Fe–O vibrational band suggest coordination interactions between Fe^{3+} ions on the NP surface and oxygen-containing functional groups, such as carboxylates or phosphates, present on the bacterial cell wall. The combined shifts observed in the –OH and Fe–O regions provide compelling evidence for chemical bonding and successful formation of the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid.

X-ray photoelectron spectroscopy (XPS) was employed to elucidate the surface composition and chemical states of the $\gamma\text{-Fe}_2\text{O}_3$ NPs and the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid. The XPS survey spectra (Fig. 1c) confirmed the presence of Fe and O in both pristine $\gamma\text{-Fe}_2\text{O}_3$ NPs and the nanobiohybrid. Notably, the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR spectrum also exhibited distinct N 1 s and C 1 s signals, absent in the pristine sample, indicating successful incorporation of LR onto the $\gamma\text{-Fe}_2\text{O}_3$ NPs. High-resolution XPS spectra for Fe, O, N, and C in both samples are shown in Fig. 1d–i. The Fe 2p core-level spectra of $\gamma\text{-Fe}_2\text{O}_3$ NPs and the nanobiohybrid (Fig. 1d, e) revealed a dominant Fe $2p_{3/2}$ peak at 711.0 eV with a corresponding satellite at 719.1 eV, along with an Fe $2p_{1/2}$ peak at 724.7 eV and its satellite at 733.7 eV. These binding energies are consistent with Fe^{3+} oxidation states and align with previously reported values for $\gamma\text{-Fe}_2\text{O}_3$ [34], confirming the phase identity and oxidation state of iron in the synthesized $\gamma\text{-Fe}_2\text{O}_3$ NPs. The O 1 s spectrum of the $\gamma\text{-Fe}_2\text{O}_3$ NPs (Fig. 1f) was deconvoluted into two components at 529.8 eV and 531.3 eV, corresponding to metal–oxygen (M–O) bonds and surface-bound H_2O , respectively [35]. In contrast, the O 1 s spectrum of the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid (Fig. 1g) displayed a broadened peak centered at 531.1 eV, which was resolved into three components: 529.5 eV (M–O bond), 531.0 eV (carbonyl $\text{O}=\text{C}-\text{H}$), and 532.3 eV (adsorbed H_2O) [36]. The presence of additional oxygen-containing functionalities in the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid suggests specific interactions between LR biomolecules and the NP surface. A key distinguishing feature of the nanobiohybrid is the appearance of an N 1 s signal, absent in pristine $\gamma\text{-Fe}_2\text{O}_3$ NPs, confirming the incorporation of nitrogen-containing biomolecules from LR. The high-resolution N 1 s spectrum (Fig. 1h) was deconvoluted into two components: a peak at 399.7 eV, corresponding to C–N bonds and amide groups, and a peak at 400.4 eV, attributed to N–H functionalities typically found in proteins [37]. These nitrogen-related signals strongly support the integration of LR onto the NP surface through electrostatic interactions. The C 1 s spectrum of the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid (Fig. 1i) exhibited three main peaks at 285.4, 286.8, and 288.5 eV, which are assigned to C–C (hydrocarbon chains), C–O (hydroxyl or ether groups), and COOH (carboxyl groups), respectively [37,38]. These signals confirm the presence of organic constituents from LR, providing further evidence of bacterial surface attachment to the NPs. Collectively, the XPS results marked by the emergence of nitrogen-related peaks, alterations in the O 1 s spectrum, and the identification of characteristic biomolecular features in the C 1 s region offer strong evidence for the successful formation of the $\gamma\text{-Fe}_2\text{O}_3$ NPs–LR nanobiohybrid. These findings highlight the role of bacterial functional groups in mediating the interaction with the NP surface.

The morphology and structure of the synthesized $\gamma\text{-Fe}_2\text{O}_3$ NPs, LR,

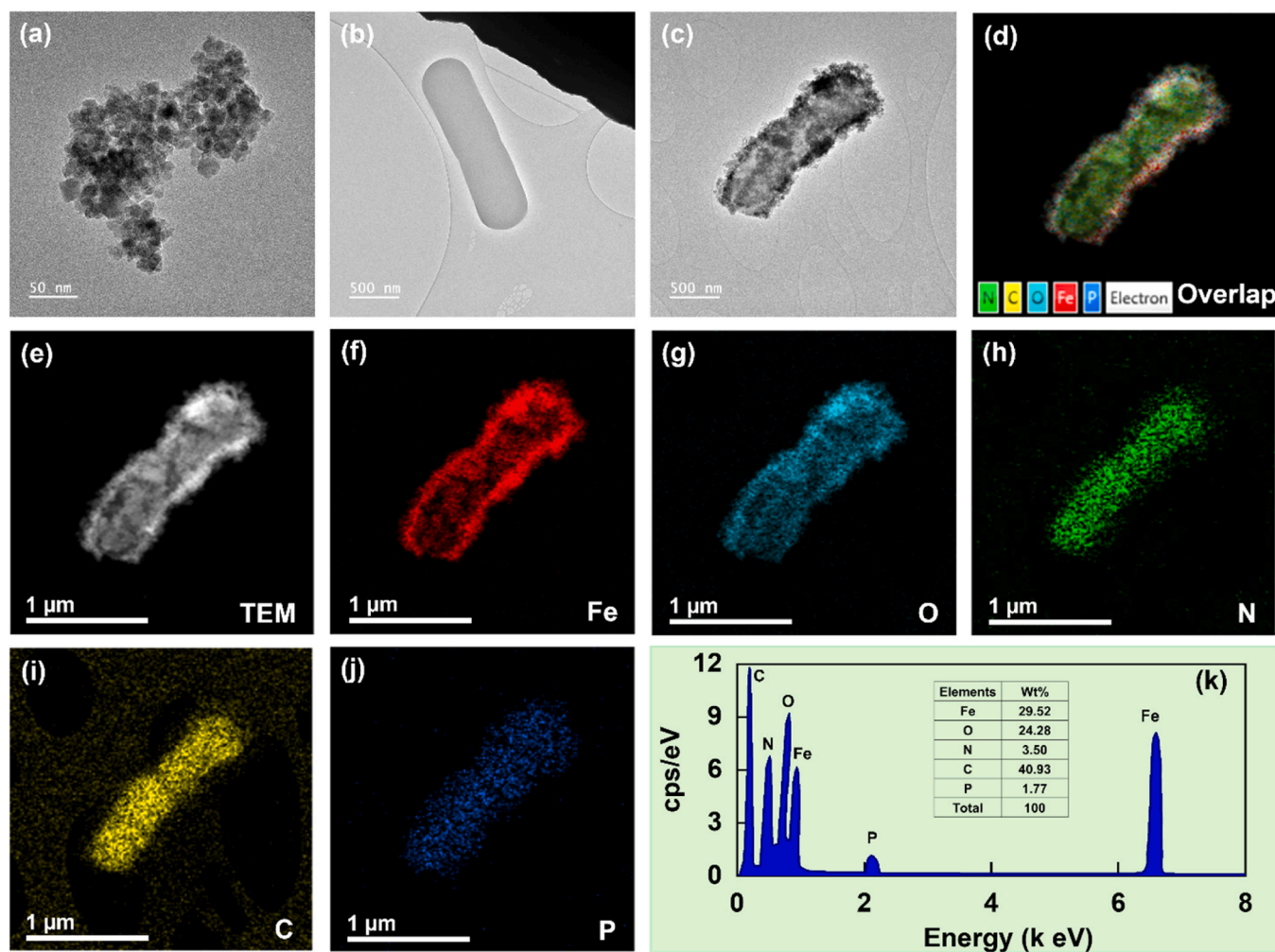


Fig. 2. TEM images of (a) γ -Fe₂O₃ NPs, (b) LR, and (c) γ -Fe₂O₃ NPs–LR nanobiohybrid. (d–j) TEM/elemental mapping and (k) EDS spectrum of the γ -Fe₂O₃ NPs–LR nanobiohybrid.

and the γ -Fe₂O₃ NPs–LR nanobiohybrid were investigated using transmission electron microscopy (TEM). The TEM micrograph of pristine γ -Fe₂O₃ NPs (Fig. 2a) revealed a well-defined spherical morphology, with average particle size of 10.5 ± 3 nm (the histogram in Fig. S1). The average particle size obtained from TEM is in good agreement with the crystallite size calculated from XRD. In contrast, the TEM image of LR (Fig. 2b) displayed its characteristic rod-like shape. Upon hybridization, TEM analysis (Fig. 2c) confirmed the successful anchoring of γ -Fe₂O₃ NPs onto the LR surface, as evidenced by the spherical NPs adhering uniformly to the bacterial cell wall. Notably, both γ -Fe₂O₃ NPs and LR retained their original morphologies following nanobiohybrid formation, indicating structural integrity and effective surface coating. Elemental composition and spatial distribution of the γ -Fe₂O₃ NPs within the nanobiohybrid were further examined via energy-dispersive X-ray spectroscopy (EDS) and elemental mapping. Elemental maps (Fig. 2d–j) confirmed the homogeneous distribution of Fe, O, N, C, and P across the γ -Fe₂O₃ NPs–LR nanobiohybrid, indicating uniform NP attachment. The corresponding EDS spectrum (Fig. 2k) showed distinct peaks for Fe, O, N, C, and P, with no detectable impurities, affirming the compositional purity of the synthesized nanobiohybrid. Collectively, the TEM and EDS analyses substantiate the successful formation of a structurally stable and compositionally uniform γ -Fe₂O₃ NPs–LR nanobiohybrid.

The morphology and size distribution of the synthesized γ -Fe₂O₃ NPs, LR, and the γ -Fe₂O₃ NPs–LR nanobiohybrid were examined using field emission scanning electron microscopy (FESEM). The FESEM

image of pristine γ -Fe₂O₃ NPs (Fig. 3a) revealed well-defined spherical NPs with sizes ranging from 6 to 17 nm, a morphology commonly observed in iron oxide NPs synthesized via the co-precipitation method [39–41]. The FESEM image of LR (Fig. 3b) displayed its characteristic rod-like morphology, typical of bacterial cells. Upon functionalization, the γ -Fe₂O₃ NPs–LR nanobiohybrid (Fig. 3c) exhibited dense and uniform adhesion of spherical γ -Fe₂O₃ NPs to the rod-shaped LR surface, indicating strong electrostatic interactions between the NPs and the bacterial cell wall. This effective attachment confirms the formation of a stable nanobiohybrid structure. The surface morphology observed via FESEM corroborates the structural features previously identified in the TEM analysis. To further evaluate particle size distribution in solution, dynamic light scattering (DLS) was conducted. The DLS profile (Fig. 3d) indicated a hydrodynamic diameter of 18.78 nm for the γ -Fe₂O₃ NPs, slightly larger than the FESEM measurements due to the presence of a hydration shell in the dispersed state. The results obtained are consistent with those reported in previous studies [42,43]. Zeta potential measurements (Fig. 3e) provided insights into the surface charge alterations during nanobiohybrid formation. The pristine γ -Fe₂O₃ NPs and LR exhibited surface charges of +22.77 and –18.44 mV, respectively. After hybridization, the zeta potential of the γ -Fe₂O₃ NPs–LR nanobiohybrid shifted to +13.06 mV, indicating successful NP binding, likely mediated by electrostatic interactions and the involvement of surface biomolecules on LR. Further validation of the hybrid formation was obtained through UV–Vis spectroscopy (Fig. 3f), shifts in the characteristic absorption peaks of both γ -Fe₂O₃ NPs and LR after conjugation

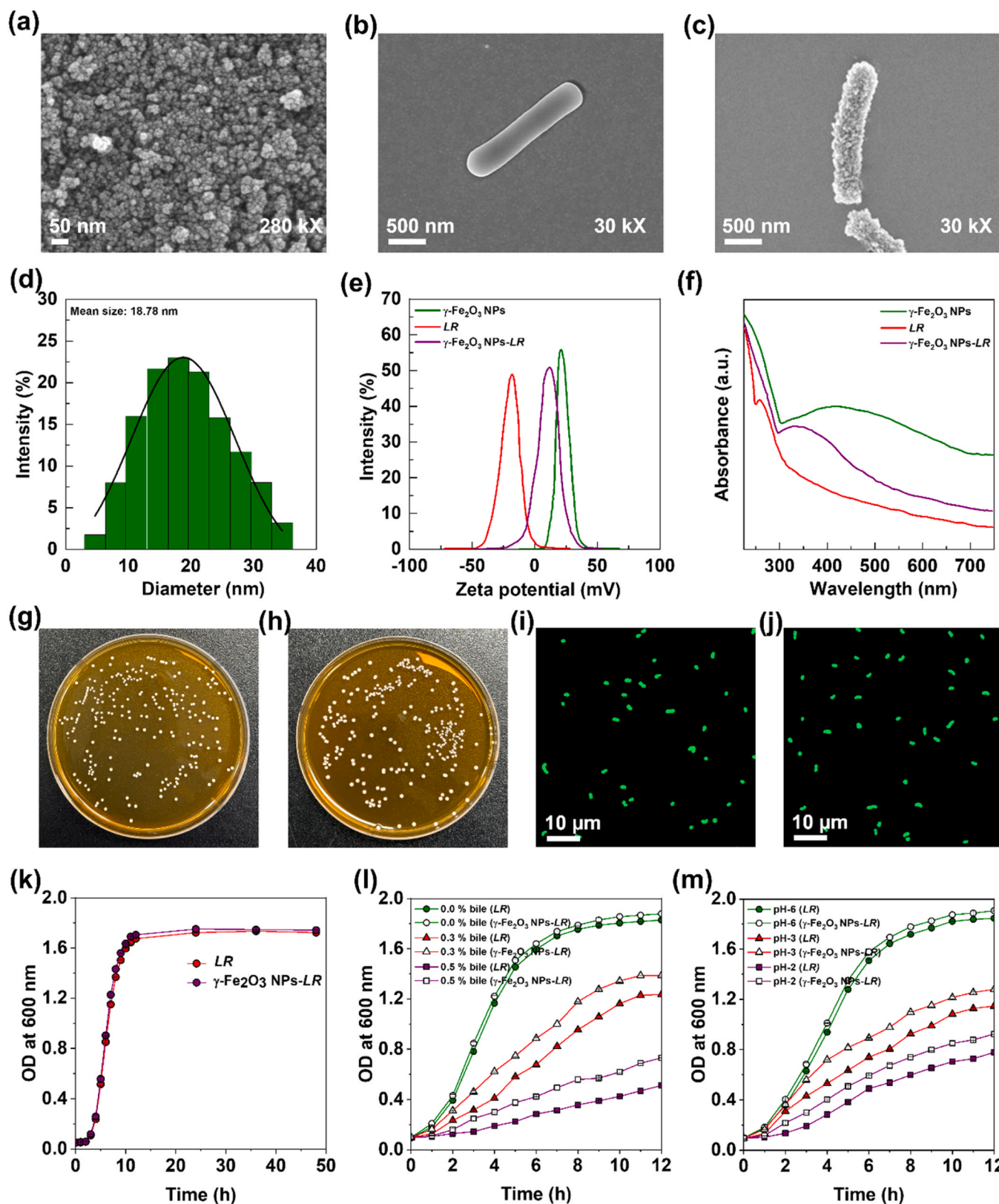


Fig. 3. FESEM images of (a) γ -Fe₂O₃ NPs, (b) LR, and (c) γ -Fe₂O₃ NPs-LR nanobiohybrid. (d) DLS profile of γ -Fe₂O₃ NPs. (e) Zeta potential and (f) UV-Vis spectra of γ -Fe₂O₃ NPs, LR, and γ -Fe₂O₃ NPs-LR nanobiohybrid. Probiotic colony morphology of (g) LR and (h) γ -Fe₂O₃ NPs-LR nanobiohybrid. CLSM images of (i) LR and (j) γ -Fe₂O₃ NPs-LR nanobiohybrid. (k) Growth curve, (l) bile tolerance, and (m) acid tolerance of LR and γ -Fe₂O₃ NPs-LR nanobiohybrid.

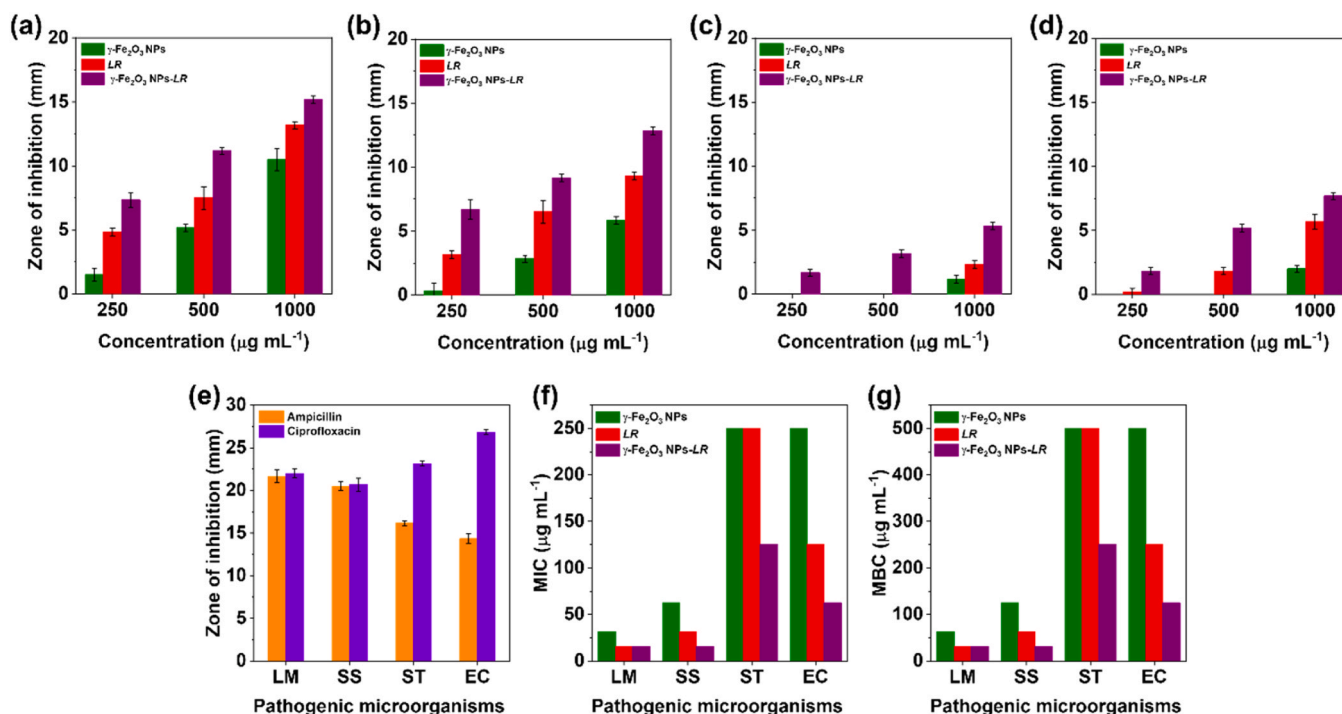


Fig. 4. Antibacterial activity of γ -Fe₂O₃ NPs, LR, and γ -Fe₂O₃ NPs-LR nanobiohybrid against (a) *L. monocytogenes*, (b) *S. succinus*, (c) *S. thompson*, and (d) *E. coli* O157:H7. (e) Antibacterial activity of Ampicillin and Ciprofloxacin against the tested pathogens. (f) MIC and (g) MBC values of γ -Fe₂O₃ NPs, LR, and γ -Fe₂O₃ NPs-LR nanobiohybrid against the tested bacterial strains.

confirmed successful nanobiohybrid assembly. A similar shift was observed in a recently reported probiotic-based hybrid [44]. These findings from FESEM, DLS, zeta potential, and UV-Vis spectroscopy strongly support the effective and stable integration of γ -Fe₂O₃ NPs onto LR, forming a well-defined nanobiohybrid structure with potential applications in biomedical fields.

3.2. Effect of γ -Fe₂O₃ NPs coating on LR

The synthesized γ -Fe₂O₃ NPs were successfully coated onto the LR surface, forming the γ -Fe₂O₃ NPs-LR nanobiohybrid. To evaluate the impact of NP attachment on bacterial viability, LR and γ -Fe₂O₃ NPs-LR were cultured on MRS agar plates, and their colony counts were compared (Fig. 3g, h). The nearly identical colony numbers indicated that γ -Fe₂O₃ NPs coating did not impair LR growth. To further verify that the coating process preserved bacterial activity, a fluorescence-based live/dead staining assay was performed. As shown in Fig. 3i, j, both unmodified LR and γ -Fe₂O₃ NPs-LR exhibited comparable green fluorescence signals, confirming that bacterial viability remained unaffected post-functionalization. Additionally, a 48 h *in vitro* proliferation assay was conducted to monitor bacterial growth kinetics (Fig. 3k). The resulting growth curves for LR and γ -Fe₂O₃ NPs-LR showed only minimal variation, reinforcing that γ -Fe₂O₃ NPs attachment does not hinder bacterial proliferation. To assess physiological stress tolerance, both formulations were exposed to varying concentrations of bile salts (0, 0.3, and 0.5%) for 12 h, and OD at 600 nm was recorded (Fig. 3l). Although both strains exhibited decreased growth with increasing bile concentrations, the γ -Fe₂O₃ NPs-LR nanobiohybrid maintained higher OD values than LR alone, suggesting that NP coating enhances resistance to bile salt-induced stress. Acid tolerance was also evaluated under pH 2.0, 3.0, and 6.0 conditions. As shown in Fig. 3m, LR alone demonstrated significantly reduced viability at pH 2.0 and 3.0 compared to γ -Fe₂O₃ NPs-LR, indicating that NP incorporation confers enhanced resistance to acidic environments. Collectively, these results demonstrate that γ -Fe₂O₃ NPs coating does not compromise LR viability or activity, but

rather improves its resilience under gastrointestinal stress conditions, positioning γ -Fe₂O₃ NPs-LR as a promising candidate for probiotic-based therapeutic applications.

3.3. Antibacterial activity

3.3.1. Agar disc diffusion method

The antibacterial activity of γ -Fe₂O₃ NPs, LR, and their nanobiohybrid (γ -Fe₂O₃ NPs-LR) was evaluated against selected pathogenic bacteria by measuring the ZOI, as shown in Fig. S2. For all tested pathogens, the γ -Fe₂O₃ NPs-LR nanobiohybrid at the highest concentration (1000 μ g mL⁻¹) exhibited greater ZOI compared to the lowest concentration (250 μ g mL⁻¹), and demonstrated superior antibacterial activity relative to γ -Fe₂O₃ NPs and LR individually. As presented in Fig. 4a–d and Tables S1–S4, the strongest antibacterial effect at 1000 μ g mL⁻¹ was observed against *L. monocytogenes* (15.17 $\pm$ 0.29 mm), followed by *S. succinus* (12.83 $\pm$ 0.29 mm), *E. coli* O157:H7 (7.67 $\pm$ 0.29 mm), and *S. thompson* (5.33 $\pm$ 0.29 mm). At 250 μ g mL⁻¹, the γ -Fe₂O₃ NPs-LR nanobiohybrid showed a ZOI of 7.33 $\pm$ 0.58 mm against *L. monocytogenes*, while the lowest inhibition was noted for *S. thompson* (1.67 $\pm$ 0.29 mm). Standard antibiotics, Ampicillin and Ciprofloxacin, were employed as positive controls to confirm the susceptibility of the pathogens, with all tested strains showing sensitivity (Fig. 4e and Table S5). The enhanced antibacterial effect of the γ -Fe₂O₃ NPs-LR nanobiohybrid suggests a synergistic interaction between the NPs and LR, resulting in improved antibacterial efficacy. The γ -Fe₂O₃ NPs are known to exert antibacterial effects primarily through the generation of ROS, which induce oxidative damage to cellular components such as lipids, proteins, and DNA, ultimately leading to bacterial cell death. Moreover, the small size and surface charge of the NPs facilitate close interaction with bacterial membranes, increasing permeability and promoting leakage of intracellular contents [45]. LR contributes to antibacterial activity through the secretion of organic acids, such as lactic and acetic acids, which lower the pH and create an inhospitable environment for pathogenic bacteria.

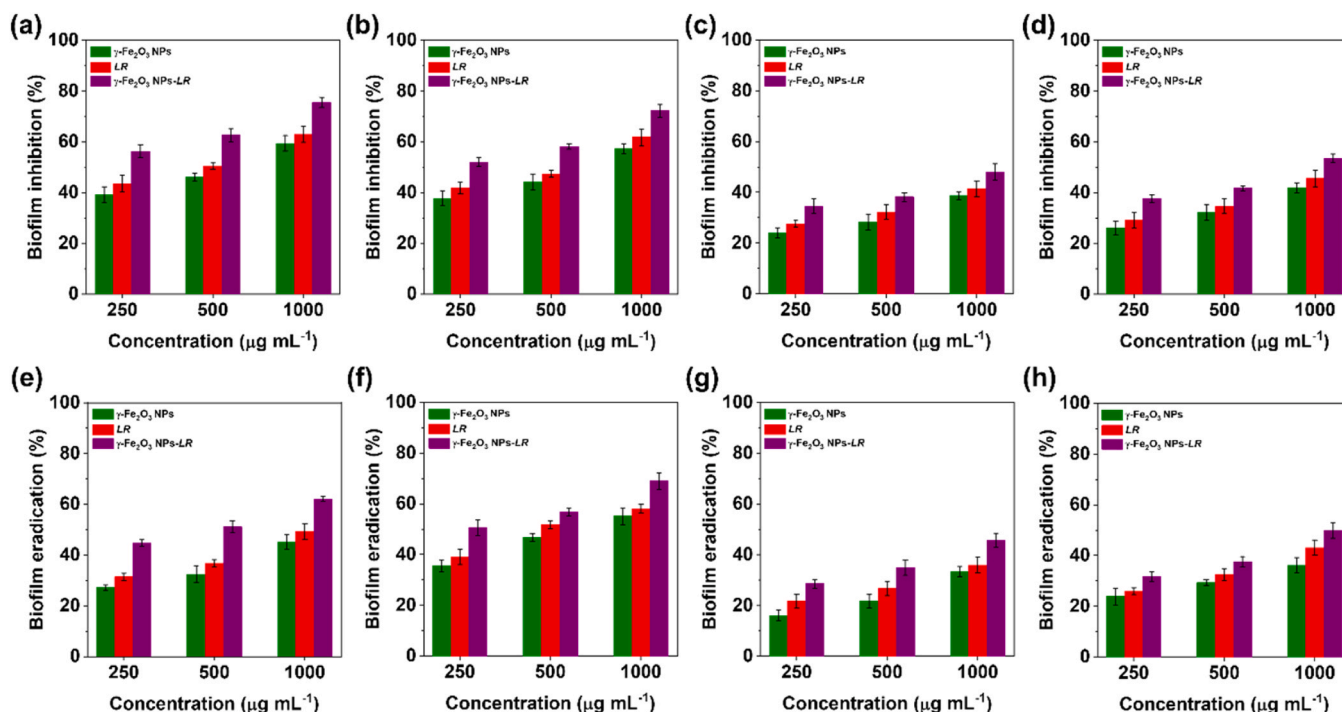


Fig. 5. Percentage of biofilm inhibition by γ -Fe₂O₃ NPs, LR, and γ -Fe₂O₃ NPs-LR nanobiohybrid against (a) *L. monocytogenes*, (b) *S. succinus*, (c) *S. thompson*, and (d) *E. coli* O157:H7. Percentage of preformed biofilm eradication by γ -Fe₂O₃ NPs, LR, and γ -Fe₂O₃ NPs-LR nanobiohybrid against (e) *L. monocytogenes*, (f) *S. succinus*, (g) *S. thompson*, and (h) *E. coli* O157:H7.

Additionally, LR is known to produce bacteriocins, proteinaceous toxins that disrupt cell wall synthesis in susceptible strains [14]. The presence of surface-associated antimicrobial peptides, along with competition for nutrients and adhesion sites, further enhances its inhibitory potential. When integrated into the γ -Fe₂O₃ NPs-LR nanobiohybrid, these mechanisms appear to act synergistically. Metabolites produced by LR may compromise bacterial membrane integrity, increasing susceptibility to ROS generated by γ -Fe₂O₃ NPs. This dual-action strategy likely facilitates deeper ROS penetration and more effective antibacterial activity. The enhanced ZOI observed with the γ -Fe₂O₃ NPs-LR nanobiohybrid supports this hypothesis, confirming that the combination of LR and γ -Fe₂O₃ NPs results in a potentiated antibacterial system with broad-spectrum efficacy. The antibacterial effects may result from the synergistic interaction of γ -Fe₂O₃ NPs-LR with bacterial cells, while the precise underlying mechanism requires further investigation in future studies. Overall, this nanobiohybrid approach represents a promising strategy for augmenting natural antibacterial defense mechanisms.

3.3.2. Broth dilution assay

The antibacterial potency of γ -Fe₂O₃ NPs, LR, and the γ -Fe₂O₃ NPs-LR nanobiohybrid was assessed by determining their MIC and MBC values against selected pathogenic bacteria. Both γ -Fe₂O₃ NPs and LR exhibited higher MIC and MBC values compared to the γ -Fe₂O₃ NPs-LR nanobiohybrid, indicating their comparatively lower antibacterial efficacy (Fig. 4f, g and Table S6). The nanobiohybrid displayed the lowest MIC value of 15.62 μ g mL⁻¹ against *L. monocytogenes* and *S. succinus*, while the highest MIC (125 μ g mL⁻¹) was observed against *S. thompson*. In terms of bactericidal activity, the MBC values for the γ -Fe₂O₃ NPs-LR nanobiohybrid were consistently lower than those of γ -Fe₂O₃ NPs or LR alone, further confirming its enhanced antibacterial efficacy. The lowest MBC (31.25 μ g mL⁻¹) was recorded against *L. monocytogenes* and *S. succinus*, whereas the highest (250 μ g mL⁻¹) was noted for *S. thompson*. These results support a synergistic interaction between γ -Fe₂O₃ NPs and LR, contributing to a multifaceted antibacterial mechanism involving membrane disruption, oxidative stress via ROS

generation, interference with protein function, and structural damage to bacterial cells [15]. The γ -Fe₂O₃ NPs-LR nanobiohybrid demonstrated broad-spectrum antibacterial activity, with heightened efficacy against Gram-positive bacteria such as *L. monocytogenes* and *S. succinus*. This increased susceptibility is likely due to the composition and higher permeability of Gram-positive cell walls [46]. These findings underscore the potential of γ -Fe₂O₃ NPs-LR as a safe and effective antibacterial agent for biomedical applications. The antibacterial activity results obtained in the present study are comparable to or superior to those reported in previous studies.

3.4. Antibiofilm activity

3.4.1. Inhibition of biofilm formation

The antibiofilm activity of γ -Fe₂O₃ NPs, LR, and the γ -Fe₂O₃ NPs-LR nanobiohybrid was evaluated against *L. monocytogenes*, *S. succinus*, *S. thompson*, and *E. coli* O157:H7 (Fig. 5a–d). Among the individual components, γ -Fe₂O₃ NPs exhibited notable biofilm inhibition, with the highest activity observed against *L. monocytogenes* (59.38 $\pm$ 3.06%) and the lowest against *S. thompson* (38.80 $\pm$ 1.56%) at 1000 μ g mL⁻¹. This activity is primarily attributed to the generation of ROS, which disrupt the biofilm matrix through oxidative damage. LR also demonstrated significant antibiofilm effects, particularly against *L. monocytogenes* (62.98 $\pm$ 3.19%), likely due to the secretion of antimicrobial metabolites, competition for surface adhesion sites, and interference with biofilm architecture via exopolysaccharide production [47]. Notably, the γ -Fe₂O₃ NPs-LR nanobiohybrid exhibited superior inhibition, with the highest activity against *L. monocytogenes* (75.60 $\pm$ 1.94%) and the lowest against *S. thompson* (48.08 $\pm$ 3.16%) at 1000 μ g mL⁻¹. The enhanced efficacy of the nanobiohybrid is attributed to the synergistic combination of ROS-mediated disruption by γ -Fe₂O₃ NPs and the antibacterial action of LR. Additionally, the nanoscale size and increased surface reactivity of γ -Fe₂O₃ NPs facilitate improved penetration and destabilization of the biofilm matrix. These findings underscore the potential of the γ -Fe₂O₃ NPs-LR nanobiohybrid as an effective strategy

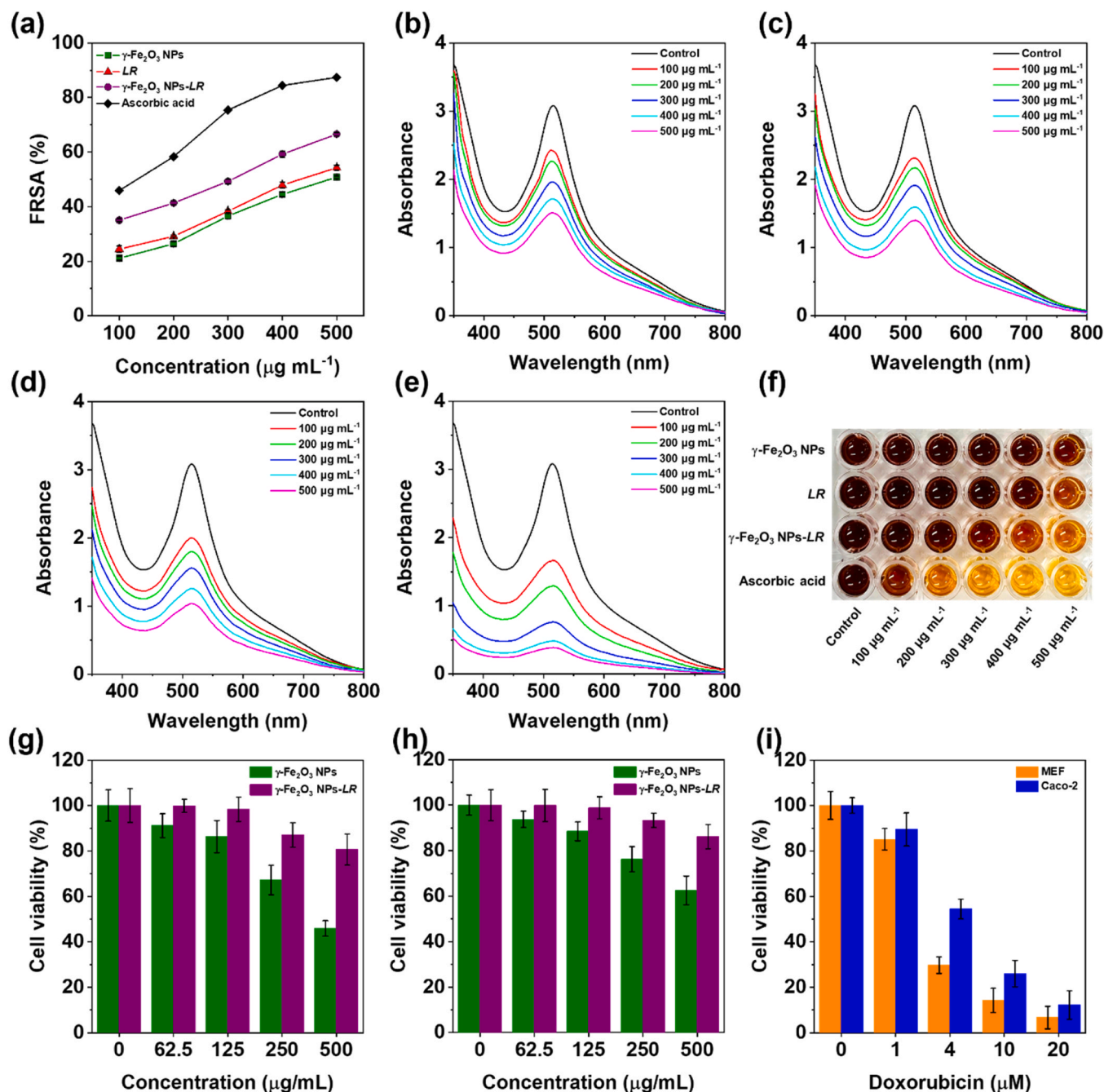


Fig. 6. Antioxidant activity of γ -Fe₂O₃ NPs, LR, γ -Fe₂O₃ NPs-LR nanobiohybrid, and ascorbic acid: (a) % FRSA; UV-Vis spectral changes of DPPH upon exposure to (b) γ -Fe₂O₃ NPs, (c) LR, (d) γ -Fe₂O₃ NPs-LR nanobiohybrid, and (e) ascorbic acid; (f) photographic representation of the DPPH assay. Cytotoxicity evaluation of (g) γ -Fe₂O₃ NPs and (h) γ -Fe₂O₃ NPs-LR on MEF and Caco-2 cell lines. (i) Cytotoxicity of DOX on MEF and Caco-2 cell lines.

for combating biofilm-associated infections, particularly those caused by Gram-positive pathogens such as *L. monocytogenes*.

3.4.2. Eradication of preformed biofilm

Eradicating established biofilms poses a greater challenge than preventing their formation due to their robust structure, which shields embedded bacteria from antibacterial agents. These biofilms consist of densely packed bacterial cells encased within an extracellular polymeric substance (EPS) matrix composed primarily of polysaccharides, proteins, and nucleic acids, which collectively hinder the penetration of antimicrobial compounds. In this study, mature biofilms of *L. monocytogenes*, *S. succinus*, *S. thompson*, and *E. coli* O157:H7 were cultivated in 96-well microtiter plates and subsequently treated with

γ -Fe₂O₃ NPs, LR, and a γ -Fe₂O₃ NPs-LR nanobiohybrid. The efficacy of each treatment in disrupting preformed biofilms was assessed using crystal violet staining. As shown in Fig. 5e–h, *S. succinus* biofilms exhibited the highest susceptibility to the γ -Fe₂O₃ NPs-LR nanobiohybrid, with a biofilm reduction of $69.02 \pm 3.21\%$ at $1000 \mu\text{g mL}^{-1}$. In contrast, *S. thompson* biofilms demonstrated greater resistance, with only a $45.70 \pm 2.85\%$ reduction at the same concentration. The γ -Fe₂O₃ NPs-LR nanobiohybrid consistently outperformed the individual treatments, indicating a synergistic effect between the ROS-mediated bactericidal activity of γ -Fe₂O₃ NPs and the biofilm-disrupting metabolites produced by LR. These findings underscore the nanobiohybrid's capacity to penetrate and disrupt mature biofilms, which are inherently resistant due to their EPS-rich architecture, thereby highlighting its

Table 1

Comparative analysis of the biological activities observed in this study with those reported in previous literature.

Nanomaterial	Concentration	Biological activity	Target bacteria	Observation	Ref.
Fe ₂ O ₃ -LAB@PVA-GA-PCL	600 µg mL ⁻¹	Disc diffusion, broth dilution, crystal violet, MTT	<i>P. aeruginosa</i> , <i>E. coli</i> , <i>C. albicans</i> , <i>S. aureus</i>	The Fe ₂ O ₃ -LAB@PVA-GA-PCL hybrid demonstrated maximum antimicrobial (24 ± 0.57) and antibiofilm activity (43%).	[15]
Fe ₃ O ₄ (IONPs)	1.25–160 µg mL ⁻¹	Agar disc diffusion, DPPH	<i>K. pneumoniae</i> , <i>P. aeruginosa</i> , <i>E. coli</i> , <i>S. aureus</i> , <i>S. sonnei</i>	The IONPs showed notable antioxidant activity against DPPH radicals, with an IC ₅₀ value of 12.2 µg mL ⁻¹ . Additionally, IONPs displayed potent antibacterial effects against <i>S. aureus</i> , producing a 12 ± 0.6 mm inhibition zone at a concentration of 250 µg.	[52]
Gallic acid-coated iron oxide NPs (IONPs-GA)	50 mg mL ⁻¹	Crystal violet, agar well diffusion	<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>K. pneumoniae</i> , <i>E. coli</i>	IONPs-GA at 50 mg mL ⁻¹ exhibited the highest antibacterial efficacy against <i>S. aureus</i> . IONPs-GA inhibited 66.86% of biofilm formation at half the MIC concentration.	[53]
Iron oxide	2.5–15 µM mL ⁻¹	DPPH, agar well diffusion	<i>K. pneumoniae</i> , <i>E. coli</i> , <i>C. xerosis</i> , <i>S. aureus</i> , <i>S. pyogenes</i> , <i>C. diphtheriae</i> , <i>P. aeruginosa</i> , <i>S. mutans</i> ,	Biogenic iron oxide NPs derived from <i>Agrewia optiva</i> demonstrated stronger antibacterial activity against Gram-positive bacteria (12.33 ± 0.33 mm against <i>S. aureus</i>). In addition, iron oxide NPs showed good DPPH activity.	[54]
MNPs-LAB	250–1000 µg mL ⁻¹	Crystal violet, agar disc diffusion, broth dilution, DPPH, WST-8	<i>S. thompson</i> , <i>E. coli</i> , <i>L. monocytogenes</i> , <i>S. succinus</i>	The MNPs-LAB hybrid demonstrated strong antimicrobial activity against <i>L. monocytogenes</i> , along with notable FRSA (71.45% at 500 µg mL ⁻¹). It effectively inhibited biofilm formation of <i>L. monocytogenes</i> (79.45% at 1000 µg mL ⁻¹) and exhibited low cytotoxicity.	[11]
Fe ₃ O ₄	100–400 µg mL ⁻¹	Tube method, Congo red agar method, agar well diffusion	<i>E. coli</i>	Fe ₃ O ₄ NPs showed strong antibiofilm activity at a concentration of 150 µg mL ⁻¹ .	[55]
Fe ₃ O ₄ -chitosan nanocomposite	0.5–100 µg mL ⁻¹	Crystal violet, agar well diffusion, broth dilution.	<i>S. maltophilia</i> , <i>S. aureus</i>	The nanocomposite inhibited biofilm formation by 72.8–85.5% and showed antibacterial zones of 21 and 13 mm against <i>S. marcescens</i> and <i>S. aureus</i> , respectively.	[56]
Probiotic		Disc diffusion agar, DPPH, crystal violet	<i>E. coli</i> , <i>S. typhimurium</i> , <i>K. aerogenes</i> , <i>S. aureus</i> , <i>S. pyogenes</i> , <i>L. monocytogenes</i>	Probiotic showed strong antioxidant potential (DPPH: 50.80%, ABTS: 56.60%, lipid peroxidation: 20.50%), notable biofilm inhibition against <i>L. monocytogenes</i> (75.50–12.50% across ¼–4 MIC).	[57]
γ-Fe ₂ O ₃ NPs-LR	250–1000 µg mL ⁻¹	Agar disc diffusion, broth dilution, DPPH, crystal violet, WST-8	<i>L. monocytogenes</i> , <i>S. succinus</i> , <i>S. thompson</i> , <i>E. coli</i> O157:H7	The γ-Fe ₂ O ₃ NPs-LR nanobiohybrid showed enhanced antibacterial activity (15.17 ± 0.29 mm at 1000 µg mL ⁻¹), inhibited biofilm formation, demonstrated superior FRSA (66.57 ± 0.53% at 500 µg mL ⁻¹), and showed low cytotoxicity in Caco-2 cells (86.19 ± 5.33%).	This study

promise as a potential therapeutic strategy for managing biofilm-associated infections.

3.5. Antioxidant activity

The antioxidant potential of γ-Fe₂O₃ NPs, LR, and their electrostatically assembled γ-Fe₂O₃ NPs-LR nanobiohybrid was evaluated using the DPPH radical scavenging assay. This method assesses the ability of antioxidants to reduce DPPH radicals, resulting in a color change from deep violet to pale yellow. Quantitative results are presented in Fig. 6a. γ-Fe₂O₃ NPs exhibited a dose-dependent increase in radical scavenging activity, with efficiency rising from 21.11 ± 0.68% at 100 µg mL⁻¹ to 50.83 ± 0.43% at 500 µg mL⁻¹. This effect is attributed to their high surface-to-volume ratio, which promotes enhanced interaction with ROS, and to the presence of Fe³⁺ ions, which contribute through redox-mediated electron donation and radical stabilization [48]. LR displayed even greater antioxidant activity, achieving 54.27 ± 0.91% scavenging at 500 µg mL⁻¹. This superior performance is primarily associated with its intrinsic biological mechanisms, including the secretion of antioxidant enzymes such as superoxide dismutase and catalase, along with bioactive metabolites such as exopolysaccharides, peptides, and organic acids. Additionally, LR's ability to chelate transition metal ions inhibits metal-catalyzed oxidative reactions, further reducing oxidative stress [49]. Remarkably, the γ-Fe₂O₃ NPs-LR nanobiohybrid exhibited the highest antioxidant activity among all tested groups, achieving a scavenging efficiency of 66.57 ± 0.53% at 500 µg mL⁻¹. This enhanced performance suggests a synergistic interaction between the inorganic γ-Fe₂O₃ NPs and LR. The γ-Fe₂O₃ NPs-LR nanobiohybrid exhibited

competitive antioxidant activity compared with reported studies, including γ-Fe₂O₃ NPs (1.18% at 20 µg) and probiotic-based systems (43.47 ± 0.66% at 8.2 × 10⁹ CFU/mL⁻¹) [48,50]. The nanobiohybrid integrates the physicochemical advantages of γ-Fe₂O₃ NPs, such as redox activity and high surface area, with the biologically driven antioxidant mechanisms of LR. As shown in Fig. 6b–e, increasing sample concentrations resulted in a progressive decrease in DPPH absorbance at 517 nm, indicating dose-dependent radical scavenging. This trend was further corroborated by the visual color change observed in Fig. 6f, where the reaction mixture shifted from violet to pale yellow with increasing concentrations, confirming efficient ROS neutralization by the nanobiohybrid system. Ascorbic acid, used as a positive control, exhibited the expected high radical scavenging activity, validating the assay's reliability. Overall, these findings underscore the potential of γ-Fe₂O₃ NPs-LR nanobiohybrids as effective antioxidant agents. The improved performance is likely due to enhanced bioavailability of γ-Fe₂O₃ NPs mediated by LR and the stabilization and potentiation of LR-derived antioxidant molecules by the inorganic matrix.

3.6. Biocompatibility assay

The biocompatibility of γ-Fe₂O₃ NPs and γ-Fe₂O₃ NPs-LR nanobiohybrid was assessed using MEF and Caco-2 cell lines treated with increasing concentrations (0–500 µg mL⁻¹) for 24 h. As shown in Fig. 6g and h, both materials induced a concentration-dependent decrease in cell viability. However, γ-Fe₂O₃ NPs alone exhibited significantly greater cytotoxicity compared to the γ-Fe₂O₃ NPs-LR nanobiohybrid. At 500 µg mL⁻¹, viability dropped to 45.89 ± 3.44% in MEF cells and

62.56 ± 6.31% in Caco-2 cells. In contrast, cells treated with the γ -Fe₂O₃ NPs–LR nanobiohybrid retained substantially higher viability (80.63 ± 6.87% in MEF and 86.19 ± 5.33% in Caco-2), indicating enhanced biocompatibility, particularly with intestinal epithelial cells. The reduced cytotoxicity of the nanobiohybrid is attributed to surface modification by LR, which creates a more biocompatible interface. Key NP characteristics such as size, morphology, surface chemistry, and concentration are known to influence cellular responses [51]. Unmodified γ -Fe₂O₃ NPs, especially at elevated concentrations, may compromise membrane integrity, disrupt intracellular processes, and induce genotoxic effects. In contrast, functionalization with beneficial microbes like LR mitigates these risks and may confer additional probiotic benefits. Moreover, the improved interaction between the γ -Fe₂O₃ NPs–LR nanobiohybrid and Caco-2 cells likely arises from the natural association of *Lactobacillus* strains with the gastrointestinal tract, further supporting its compatibility with intestinal epithelium. The assay's reliability was confirmed using DOX as a positive control, which produced a marked reduction in cell viability across both cell lines (Fig. 6i). These findings position the γ -Fe₂O₃ NPs–LR nanobiohybrid as a safe and multifunctional platform with strong potential for biomedical applications. A comparative summary of the biological activity data alongside previous studies is provided in Table 1. Although the *in vitro* results provide useful preliminary information, one limitation of this study is the lack of *in vivo* biocompatibility and toxicological evaluation, which will be addressed in future studies.

4. Conclusions

A novel γ -Fe₂O₃ NPs–LR nanobiohybrid was synthesized via a simple electrostatic self-assembly method. The γ -Fe₂O₃ NPs–LR exhibited enhanced antibacterial activity against *Listeria monocytogenes* (ZOI 15.17 ± 0.29 mm at 1000 µg mL⁻¹) and strong antibiofilm performance of 75.60 ± 1.94% inhibition. In addition, the γ -Fe₂O₃ NPs–LR demonstrated superior antioxidant activity (66.57 ± 0.53% FRSA at 1000 µg mL⁻¹) compared to γ -Fe₂O₃ NPs and LR alone. The improved performance is attributed to the synergistic interaction between γ -Fe₂O₃ NPs and LR. Importantly, the cytotoxicity of γ -Fe₂O₃ NPs–LR was significantly lower in Caco-2 cells compared to γ -Fe₂O₃ NPs alone. The γ -Fe₂O₃ NPs–LR combines high functional performance with low cytotoxicity and holds strong potential for biomedical applications, which may pave the way for the development of multifunctional agents that are both effective and biocompatible.

CRedit authorship contribution statement

Patil Abhinandan R: Writing – review & editing, Supervision, Funding acquisition. **Jong Pil Park:** Writing – review & editing, Supervision, Funding acquisition. **Padalkar Navnath S:** Methodology, Data curation. **Shingade Jayshri A:** Writing – original draft, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2026.02.009.

Data availability

Data will be made available on request.

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