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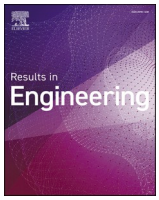

Research paper

Eco-friendly zinc nanoparticles biosynthesized by *Lactobacillus casei* as alternative anticoccidial agent ameliorate *Eimeria tenella* infection in broiler chickens: Impact on oxidative stress, intestinal health, growth performance, and gut microbiota

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ABSTRACT

Coccidiosis, a parasitic infection caused by *Eimeria* species poses significant challenges to poultry farms worldwide. Despite extensive control efforts, the disease continues to impact poultry health and productivity. Recently, green-synthesized nanoparticles have emerged as a promising area in nanotechnology for combating infections. This study evaluates the potential of *Lactobacillus casei* EE12-mediated zinc nanoparticles (Lc-ZnNPs) as an alternative anticoccidial agent in *Eimeria tenella*-infected broiler chickens and assesses their effects on growth performance, oxidative status, immunity, gut microbiota, and inflammatory responses. Zinc nanoparticles fabricated by the *Lactobacillus casei* EE12 supernatant were spherical, 41 nm, -24.4 mV charged, and surrounded with active groups. A total of 360 broiler chickens were equally divided into 6 groups: C) the control received basal diet; T1-T3) received basal diet supported with Lc-ZnNPs at different concentrations (25, 50, and 75 mg/kg diet); T4) *E. tenella*-infected group were infected orally through a stomach tube, with 5×10^4 sporulated oocysts of *Eimeria tenella*, and T5) *E. tenella*-infected broilers supplemented with Lc-ZnNPs (75 mg/kg diet). Adding Lc-ZnNPs to the broiler diet significantly improved growth performance markers, including body weight gain (BWG), feed intake (FI) & feed conversion ratio (FCR) compared to the control and infected groups. The *Eimeria*-infected broilers showed higher levels of AST, ALT, uric acid, total cholesterol, MDA, & LDL. Meanwhile, the addition of Lc-ZnNPs (75 mg/kg) mitigated the oxidative stress by lowering MDA and enhancing the activity of SOD, CAT, & GPx besides the immunity markers. Also, downregulating the intestinal, hepatic, & kidney proinflammatory markers (Mucin-1, OCCU & interleukin-6, & IL-1β) and proapoptotic (Bcl-2-associated protein x & Casp-3) markers. Lc-ZnNPs significantly ($P < 0.05$) reduced oocyst per gram (OPG) by 67 % and lesion score, recovering the typical structure of intestines. The *E. tenella*-infected broilers showed higher total bacterial count (TBC), total yeasts and molds count (TYMC), and *Salmonella* count; meanwhile, the Lc-ZnNPs-treated birds in T5 significantly ($P < 0.05$) reduced these counts and enhanced the lactic acid bacteria (LAB) count compared to the T4 and control group. In conclusion, the findings indicated that Lc-ZnNPs could be a safe and potent anticoccidial agent against coccidiosis in broiler chickens.

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1. Introduction

Avian coccidiosis, caused by the parasite *Eimeria tenella*, inflicts severe economic losses in poultry production worldwide, with annual global costs estimated in the billions of dollars due to reduced weight gain, increased mortality, and treatment expenses [1]. This intracellular pathogen invades the intestinal epithelial cells of chickens, leading to destructive infections that impair nutrient absorption, trigger debilitating intestinal hypermotility, and induce hemorrhagic enteritis [2]. The resulting growth retardation, higher feed conversion ratios (FCR), and flock mortality significantly diminish farm profitability. The uncontrolled coccidiosis compromises poultry welfare and productivity, making it one of the industry's most costly and pervasive challenges [3]. Different *Eimeria* species exhibit varying degrees of virulence and target specific regions of the intestine, resulting in diverse clinical signs [4]. For example, *Eimeria tenella* primarily affects the cecum, causing severe hemorrhagic lesions, while *Eimeria acervulina* targets the upper small intestine [5]. The lifecycle of *Eimeria* is complex, involving both asexual and sexual reproduction within the host, culminating in the shedding of environmentally resistant oocysts [6]. When ingested by susceptible birds, these oocysts initiate a new cycle of infection [7]. The *Eimeria* infection harms the intestines, causing severe inflammation and increased oxidative stress [8], and indirectly impacts the gut microbiota and other organs, such as the liver [9,10]; precisely, impaired hepatic function and tissue damage were evidenced by elevated liver enzyme levels, including ALT, AST, ALP, γ-glutamyl transferase, and total bilirubin [9,11]. The disease's clinical progression encompasses a variety of symptoms, including weight loss, diarrhea, stunted development, reduced appetite, and, in extreme cases, death [12].

The severity of coccidiosis is influenced by several factors, including the species of *Eimeria*, the dose of oocysts ingested, the bird's age and immune status, and environmental conditions [13]. Control measures are crucial for mitigating the impact of coccidiosis on poultry production [14]. Traditionally, anticoccidial drugs have been widely used for both prophylactic and therapeutic purposes. These drugs, categorized as ionophores or synthetic compounds, target different stages of the Eimeria life cycle [15]. Ionophores, such as monensin and salinomycin, disrupt ion transport across parasite membranes, while synthetic compounds, like toltrazuril and diclazuril, interfere with parasite metabolism [16]. However, the widespread and often indiscriminate use of anticoccidial drugs has led to the emergence of drug resistance, posing a significant challenge to effective coccidiosis control. Resistance development is attributed to genetic mutations in the parasite, coupled with selective pressure exerted by continuous drug exposure. This has resulted in reduced efficacy of several commonly used anticoccidial drugs, necessitating the development of alternative control strategies [17–19]. Vaccination has emerged as a promising alternative, offering a more sustainable approach to coccidiosis control. Live attenuated or live virulent vaccines and subunit vaccines stimulate the host's immune system to develop protective immunity against *Eimeria* challenge. Live vaccines, containing attenuated or virulent oocysts, induce a controlled infection, priming the immune system for subsequent challenges [20]. Subunit vaccines, on the other hand, utilize specific *Eimeria* antigens to elicit targeted immune responses [21]. However, the development and application of vaccines are influenced by factors such as the cost of production, the complexity of administration, and the availability of serotype-specific vaccines [22]. Integrated control strategies are essential for sustainable coccidiosis control, combining judicious use of anticoccidial drugs, vaccination, and improved management practices. Recent research highlights the importance of gut microbiota modulation, nutritional interventions [23], and natural products, such as plant extracts and prebiotics, as adjunct therapies to enhance poultry resilience against coccidiosis [24–26]. These alternatives aim to improve gut health, strengthen the immune system [27,28], and reduce reliance on conventional anticoccidial drugs.

Recently, nanotechnology has emerged as a promising tool in the veterinary field, offering new possibilities for diagnosing diseases, developing therapies, and implementing prevention strategies [29]. Nanoparticles provide significant advantages in biological applications due to their tiny size and unique physicochemical properties. These features enable precise, controlled drug delivery and allow for the manipulation of the immune system within living organisms [30–32]. Moreover, elemental nanoparticles have demonstrated considerable antibacterial, anti-parasitic, and antioxidant properties [33].

Zinc nanoparticles (ZnNPs) are inorganic salts characterized by particle dimensions ranging from 1 to 100 nanometers. These nanomaterials can be synthesized through various methodologies, including chemical precipitation, physical vapor deposition, and environmentally benign "green" synthesis routes [34,35]. Microbial-mediated zinc nanoparticle synthesis is more eco-friendly and environmentally safe than chemical and physical methods [36,37]. ZnNPs have garnered significant attention due to their distinctive physicochemical properties, including tailored morphology, nanoscale dimensions, enhanced surface area and reactivity, notable catalytic capabilities, and pronounced adsorption capacities [38,39]. Furthermore, ZnNPs have wide-spectrum antimicrobial properties against many pathogens [40,41]. Therefore, including ZnNPs as a feed additive in chickens' diets can improve growth performance and induce their immunity [42]. Additionally, zinc possesses antioxidant properties, is essential for the antioxidant defense system activity [43], and is a component of various proteins and enzymes acting as immune protection mechanisms, secondary metabolites, and hormone secretion pathways [44].

Despite the implementation of effective management strategies, advancements in breeding methodologies, and the application of sterilizing agents aimed at preventing the ingestion of sporulated oocysts by young chicks, coccidiosis persists as a significant contributor to substantial economic losses within the global poultry industry [45]. ZnO—NPs could be a promising alternative in cases of anticoccidial drug resistance, where the commercial zinc oxide nanoparticles at 20, 40, and 60 mg/kg were tested against *E. tenella* oocysts isolated from infected broilers. 60 mg/kg ZnO—NPs was the most effective, significantly reducing oocyst shedding and mortality (0.8 %) compared to controls and the amprolium-treated group [46]. Meanwhile, the green zinc nanoparticles (60 mg/kg) from *Nigella sativa* extract improved growth performance, reduced oocyst shedding, and enhanced the anticoccidial index [47]. To address existing limitations in the literature, this work investigates the antioxidant and anti-parasitic efficacy of biosynthesized zinc nanoparticles using *Lactobacillus casei* EE12 supernatant (Lc-ZnNPs). The study also evaluates their potential as a natural alternative to chemical anticoccidials and assesses their effects on the immune response, histological alterations, and growth performance in broiler chickens challenged with *Eimeria tenella*.

2. Materials and methods

2.1. Isolation of zinc-tolerant bacteria

Lactobacillus casei EE12 was isolated from dairy milk samples (yoghurt, Domiati cheese, and fermented milk); for isolation, serial dilutions were prepared; 10 mL of the homogenized sample was aseptically transferred to 90 mL of saline peptone buffer (0.1 % peptone, 0.85 % NaCl), yielding a 10^{-1} dilution. Subsequently, 1 mL of this initial dilution was transferred to a 9 mL saline peptone buffer to achieve a 10^{-2} dilution. This process was serially repeated up to a 10^{-7} dilution. 100 μL of each dilution were spread-plated in triplicate onto the surface of selective agar media within sterile 90 mm plastic Petri dishes using sterile L-shaped spreader. For the selective isolation of zinc-tolerant *Lactobacillus* species, de Man Rogosa and Sharpe (MRS) agar (CM0361, Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) supplemented with Zinc Nitrate [$Zn(NO_3)_2{\cdot}6H_2O$] at different concentrations of 1, 2, 3, 4, and 5 mM were employed. The developed colonies at 5 mM were purified, and identified by MALDI-TOF as stated in Aioub et al. [48].

2.2. Biosynthesis and characterization of zinc nanoparticles (Lc-ZnNPs)

Lactobacillus casei EE12 was grown in 100 ml of Luria-Bertani broth at 37 °C with shaking incubator at 30 °C/180 rpm for 96 h until it reached the log phase (1.5×10^8 CFU/mL). Following centrifugation at 6500 × g for 20 min, the resulting bacterial supernatant was collected, and 20 ml of the bacterial supernatant was combined with 80 mL of 5 mM zinc nitrate, then incubated at 30 °C with shaking incubator at 30 °C/180 rpm for 96 h, the flask color turned colorless to white, indicating that the *Lactobacillus casei* EE12 isolate was transformed the zinc nitrate into Lc-ZnNPs, which were subsequently collected by centrifugation at 10,000 × g for 15 min, washed, and lyophilized [49,50].

The Lc-ZnNPs were characterized using UV and Transmission Electron Microscopy to reveal the particle shape. The particle size distribution was assessed using the Zeta sizer, while the surface charge was evaluated using Zeta potential. The active groups in the Lc-ZnNPs identified using Fourier-transform infrared spectroscopy (FTIR); this technique generated an infrared absorption spectrum, which provides a detailed analysis of the molecular structure and composition of the nanoparticles.

2.3. Antioxidant activity of Lc-ZnNPs

The DPPH scavenging activity of Lc-ZnNPs was assessed following Abdel-moneim et al. [51]. 0.5 mL of ethanolic DPPH was added to 1 mL of Lc-ZnNPs at different concentrations (25, 50, 75, and 100 μg/mL), then incubating for 30 min in the dark, the absorbance was determined spectrophotometrically at 517 nm and calculated in the following equation:

$$\%\ DPPH\ scavenging\ activity = \frac{Abs\ control - Abs\ sample}{Abs\ control} x100 \quad (1)$$

The IC_{50} value was the minimum Lc-ZnNPs concentration that scavenged 50 % of the DPPH radicals [52].

2.4. Antibacterial activity of Lc-ZnNPs

Staphylococcus aureus, Bacillus cereus, Salmonella typhi, & Escherichia coli were used in this study to evaluate the antibacterial activity of Lc-ZnNPs using a disc assay method [53]. The Muller-Hinton agar (MHA) was added to the plates, and then 100 μL of bacterial inoculum was spread over the plates' surface. Paper discs (8 mm) were previously immersed in Lc-ZnNPs at different concentrations (25, 50, 75, and 100 μg/mL) and were seeded on each plate surface. The negative control discs were soaked with sterilized distilled water. The MHA plates were incubated for 24 h at 37 °C [54]. Diameters of the inhibition zones (mm) were measured [55]. The minimum inhibitory concentration (MIC) values were estimated as mentioned in Saad et al. [56].

2.5. Anticoccidial activity of Lc-ZnNPs

Lc-ZnNPs concentrations (25, 50, 75, and 100 μg/mL) were prepared in Dulbecco's modified eagle medium (DMEM; HyClone, USA) supplemented with 5 % FBS. The antiococcial agent of Lc-ZnNPs were estimated by Cell Counting Kit-8 (CCK-8) assay. Embryo fibroblast Cells were seeded in 96-well plates and incubated for 24 h. Following washing, cells were treated with various concentrations of Lc-ZnNPs or a control solution (DMEM with 5 % FBS). After 24 and 48 h of treatment, CCK-8 solution was added to each well, and absorbance was measured at 450 nm to determine cell viability. All treatments were performed in triplicate [57].

2.6. Experimental design

Chickens' husbandry and diets: A total of 360 one-day-old broiler chicks were reared in separate pens following the National Research Council (NRC) recommendations. From days 1 to 10, they were fed a basal diet containing 24 % crude protein and 2900 kcal ME/kg [58]. The ingredient composition and nutritional profile of both the basal and experimental diets are detailed in Table 1.

Eimeria tenella oocyst preparation: *E. tenella* oocysts were isolated using flotation, washed with tap water, and centrifuged at 2500 × g for 10 min. The oocysts were suspended in a 2.5 % potassium dichromate solution to induce sporulation and incubated for 72 h at room temperature. After sporulation, the oocysts were rinsed with phosphate buffer saline, centrifugated at 2500 × g, 10 min, and the supernatant was obtained.

Infection protocol: Birds were orally inoculated via stomach tube with sporulated *E. tenella* oocysts (5×10^4) per chick. The infective dose was standardized to 2.5×10^4 oocysts/bird using the McMaster counting technique [2,59].

Experimental groups: The 360 broilers were randomly allocated into six groups ($n = 60$/group, 6 replicates of 10 birds each):

Group C (Negative Control): Basal diet, no additives, no infection; Groups T1–T3: Basal diet supplemented with 25, 50, and 75 mg Lc-ZnNPs/kg, respectively; Group T4 (Positive Control): *E. tenella*-infected and basal diet only; Group T5: *E. tenella*-infected, basal diet + and treated with 75 mg Lc-ZnNPs /kg.

Sample collection and analysis: On day 14 (challenge day), 7 days post-infection (dpi), and 14 dpi, three birds per group were humanely euthanized for sample collection. The intestinal of infected birds was examined for parasite stages (oocysts/cysts) at each time point. Additionally, excreta were collected and analyzed for oocyst shedding [2].

2.7. Growth performance

Birds were weighed individually at the beginning of the experiment. Feed intake (FI, g) was reported through the growth period (10–35 days

Table 1
Structure and calculated analysis of basal & experimental ration of broiler's ingredients (%).

Diets	Starter period (1–21 days)	Grower period (22–35 days)
Ingredients%		
Yellow corn	55.50	60.50
Soybean meal (46 %)	26.25	21.50
Full fat soya	5.00	6.00
Gluten (60 %)	7.00	6.00
Soybean oil	1.75	1.50
Mono calcium phosphate	1.65	1.65
Limestone	1.75	1.75
L-lysine	0.25	0.25
DL–methionine	0.20	0.20
Salt (NaCl)	0.35	0.35
Premix*	0.30	0.30
Total	100	100
Calculated analysis		
Crude protein,%	23.00	21.14
ME (kcal/kg)	3059	3101
Crude fiber,%	3.83	3.59
Ether extract,%	5.23	5.28
Calcium,%	1.06	1.05
Phosphorus available,%	0.45	0.45
Methionine,%	0.56	0.54
Lysine,%	1.23	1.13
Methionine + Cystine,%	0.95	0.90

* Every kg of mineral & vit. mix has 12 M IU vitamin A; 5 M IU D3; 80,000 mg E; 4000 mg K; 4000 mg B1; 9000 mg B2; 4000 mg B6; 20 mg B12; 15,000 mg pantothenic acid; 60,000 mg Nicotinic acid; 2000 mg Folic acid; 150 mg Biotin; 400,000 mg Choline Chloride; 15,000 mg Copper sulfate; 1000 mg calcium Iodide; 40,000 mg ferrous sulfate; 100,000 mg Manganese oxide; 100,000 mg Zinc oxide and 300 mg Selenium selenite.

of age). The uneaten feed was discarded, and the body weight gain (BWG, g), feed conversion ratio (FCR), growth rate (GR), and performance index (PI) were calculated using the following equations [60]:

$$BWG10-35 = BW35 - BW10 \quad (2)$$

$$FCR = FI\ (g)/\ BWG(g) \quad (3)$$

$$GR = \frac{(LBW35 - LBW10)}{0.5 \times (LBW35 + LBW10)} \quad (4)$$

$$PI = \frac{BWG\ (g)}{FCR} \quad (5)$$

2.8. Blood biochemistry

At 35 days old, blood samples were collected from slaughtered birds to assess biochemical parameters: ALT, AST, uric acid, & creatinine [61,62]. Meanwhile, the antioxidant markers, such as SOD, MDA, CAT, & GPx, were estimated in the serum using commercial kits [63–65].

2.9. Gene expression

RNA extraction and quality assessment: Total RNA was extracted from broiler tissues using Invitrogen™ Trizol reagent (Thermo Fisher Scientific). RNA purity was assessed by measuring the A260/A280 ratio with a NanoDrop ND-2000C spectrophotometer, using 1.5 μL of RNA per sample [66]. cDNA synthesis: Fifty nanograms of RNA were reverse-transcribed into cDNA in a 20 μL reaction volume using the cDNA Reverse Transcription Kit (Applied Biosystems). The reaction mixture consisted of 5 μL of nuclease-free water, 4 μL of 5× miRCURY RT reaction buffer, 2.5 μL of 10× miRCURY RT Enzyme Mix, and 1.2 μL of gene-specific primer. The mixture was incubated at 42 °C for 60 min for reverse transcription, followed by enzyme inactivation at 85 °C for 10 min in a Biometra 96-well thermal cycler (Applied Biosystems). The synthesized cDNA was aliquoted and stored at –20 °C until further use.

Quantitative Real-Time PCR (qRT-PCR): qRT-PCR was performed on a Rotor-Gene Q 2plex Real-Time PCR System (Qiagen, Germany) using TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (Enzynomics, Korea; Cat. # P725 or P750). The cycling conditions were as follows: Initial denaturation: 95 °C for 12 min, 40 cycles of Denaturation: 95 °C for 20 s, Annealing: 60 °C for 30 s, and Extension: 72 °C for 30 s.

Gene-specific primers (Sangon Biotech, Beijing, China) were used for amplification. Target gene expression was normalized to β-actin mRNA as an internal control, and relative quantification was performed using the $2^{-\Delta\Delta CT}$ method [67].

2.10. Histopathological investigation

Intestinal tissues were collected, fixed in 10 % formalin for 48 h, and then processed using an automated tissue processor. Following fixation, tissues were washed in distilled water for 30 min and then dried using different immersions in alcohol with various concentrations (70 % for 120 min, 90 % for 90 min, and 98 % for 90 min). Then, the tissues were submerged in xylene (50 %) for 60 min, alcohol (50 %), and pure xylene for an additional 90 min. The tissues were covered with melted paraffin wax, sealed, then paraffin cut into specimens (4–5 μm), and then stained with Hematoxylin & Eosin [68].

2.11. Microbial analysis

Sample collection: At 35 days of age, three birds per replicate (nine per group) were euthanized for intestinal content collection. Intestinal contents were aseptically transferred to sterile cups and stored at 4 °C until microbial analysis.

Microbial dilution & plating: samples were serially diluted to 10^{-6}, and 100 μL of hommoginized sample were spread onto agar plates using a sterile glass rod. The following media were used for selective enumeration:

Total aerobic bacteria on nutrient agar (Lab M, LAB008), Total yeasts and molds count on Sabouraud dextrose agar (HiMedia, MH063), *Escherichia coli* on eosin methylene blue (EMB) agar (Lab M, LAB061), *Salmonella* spp. on xylose lysine decarboxylase (XLD) agar (Lab M, LAB032), and Lactic acid bacteria (LAB) on MRS agar (Lab M, LAB223)

Incubation & quantification: Total aerobic bacteria: 30 °C for 1 day. *E. coli*, and *Salmonella*: 37 °C for 1 days. Lactic acid bacteria: 37 °C for 3 days. Total yeasts and molds: 28 °C for 5 days. The microbial counts were estimated as colony-forming units per gram (CFU/g) sample [69].

2.12. Statistical analysis

Pretests were carried out before a one-way analysis of variance (ANOVA) was performed. The Levene test with a *P*-value of 0.01662 was used to determine the homogeneity and to evaluate the normality assumption on sample distributions.

The data were presented as mean ±SD. The triplicate data were analyzed by SPSS (version 16.0, SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to compare the means of the three data sets with a 95 % confidence level [70]. Eq. (6) was used to determine the sample size.

$$n = \frac{(ZSD)^2}{E} \quad (6)$$

n = required sample size; Z = *Z*-score (standard normal deviate corresponding to desired confidence level), S = standard deviation (a measure of variability in the population), D = design effect (accounts for clustering if present, typically 1 for simple random sampling), E = margin of error.

3. Results

3.1. Biosynthesis and characterization of Lc-ZnNPs synthesized by *Lactobacillus casei* EE12

Thirty-eight bacterial isolates were isolated from dairy samples were screened for zinc tolerance on MRS plates supplemented with zinc nitrate [Zn $(NO_3)_2{\cdot}6H_2O$] 1–5 mM coded as (EE1, EE2, EE3, ….and EE38). 20 isolates were survived at MRS plates supplemented with 1 and 2 mM of zinc nitrate, 11 isolates were at MRS plates supplemented with 3 mM of zinc nitrate, and 6 isolates survived at MRS plates supplemented with 4 mM zinc nitrate. Only one isolate (EE12) could grow at MRS plates supplemented with high zinc nitrate concentration (5 mM). This isolate was identified as a Gram-positive, catalase-negative, non-spore-forming, and homo-fermentative bacterium, consistent with *Lactobacillus* spp., based on the tests' results, it was nominated as *Lactobacillus casei* EE12. MALDI-TOF mass spectrometry confirmed the isolate as *Lactobacillus casei* EE12, showing 99 % similarity to *Lactobacillus* spp. and high similarity to *Lacticaseibacillus casei* DSM 20,011.

Lactobacillus casei EE12 was cultured in LB broth for 96 h until log phase (1.5×10^8 CFU/mL). The supernatant (20 mL), after centrifugation, was mixed with 5 mM zinc nitrate (80 mL) and incubated at 30 °C with shaking for 96 h. The color change to white indicated the formation of Lc-ZnNPs by the bacteria. Ultraviolet-visible (UV–Vis) spectroscopy of the synthesized Lc-ZnNPs revealed a characteristic absorption peak at 365 nm (Fig. 1A). Transmission Electron Microscopy (TEM) analysis (Fig. 1B) demonstrated that the nanoparticles possessed a spherical shape. Zeta sizer analysis determined a mean hydrodynamic diameter of 41 nm, while zeta potential measurements indicated a surface charge of −24.4 mV (Fig. 1C and D). Fourier-transform infrared spectroscopy (FT-IR) was employed to analyze the composition and formation of functional groups within the synthesized Lc-ZnNPs (Fig. 1F). The FT-IR spectra, spanning 400–4000 cm^{-1}, revealed that the formation of Lc-ZnNPs was mediated by interactions with various plant-derived

compounds, including phenolic compounds, alkynes, terpenoids, and flavonoids. Specific functional groups, identified by their characteristic absorption bands, were crucial in reducing zinc ions to Lc-ZnNPs. A broad band at 3420 cm^{-1} indicated O—H stretching in phenolic compounds. A peak confirmed the alkene group at 1630 cm^{-1}, and C—N stretching of amines was observed at 1320 cm^{-1}. C—O stretching of esters and carboxylic groups was detected between 1025 and 1300 cm^{-1}. Most importantly, distinct sharp bands at 510 cm^{-1} and 450 cm^{-1} confirmed the presence of C-I stretching, validating the successful synthesis of ZnNPs.

3.2. Antioxidant activity of Lc-ZnNPs

Fig. 2 shows that Lc-ZnNPs have significant ($P < 0.05$) scavenging activity against DPPH free radicals. The findings displayed a positive relation between the different levels of Lc-ZnNPs & their antioxidant ability, eliminating 90 % of DPPH radicals at *Lc*-ZnNPs concentration (100 μg/mL); meanwhile, The IC_{50} of Lc-ZnNPs was 55 μg/mL.

3.3. Antibacterial activity of Lc-ZnNPs

Fig. 3 shows that all the tested Lc-ZnNPs levels (25, 50, 75, & 100 μg/mL) have a significant antibacterial activity ($P < 0.05$) against the tested pathogenic bacteria in the present study (*Staphylococcus aureus, Bacillus cereus, Salmonella typhi, & Escherichia coli*). The antibacterial activity of Lc-ZnNPs increased ($P < 0.05$) in a concentration-dependent manner.

Staphylococcus aureus exhibited the highest sensitivity to *Lactobacillus casei*-derived zinc oxide nanoparticles (Lc-ZnNPs), demonstrating an inhibition zone diameter (IZD) of 37 mm at a 100 μg/mL concentration. *Bacillus cereus* displayed the highest sensitivity, with an IZD of 31 mm under the same conditions. Conversely, *Salmonella typhi* showed the greatest resistance to Lc-ZnNPs, yielding an IZD of 25 mm, followed by *Escherichia coli* with an IZD of 28 mm. The minimum inhibitory concentration (MIC) values of Lc-ZnNPs against the tested

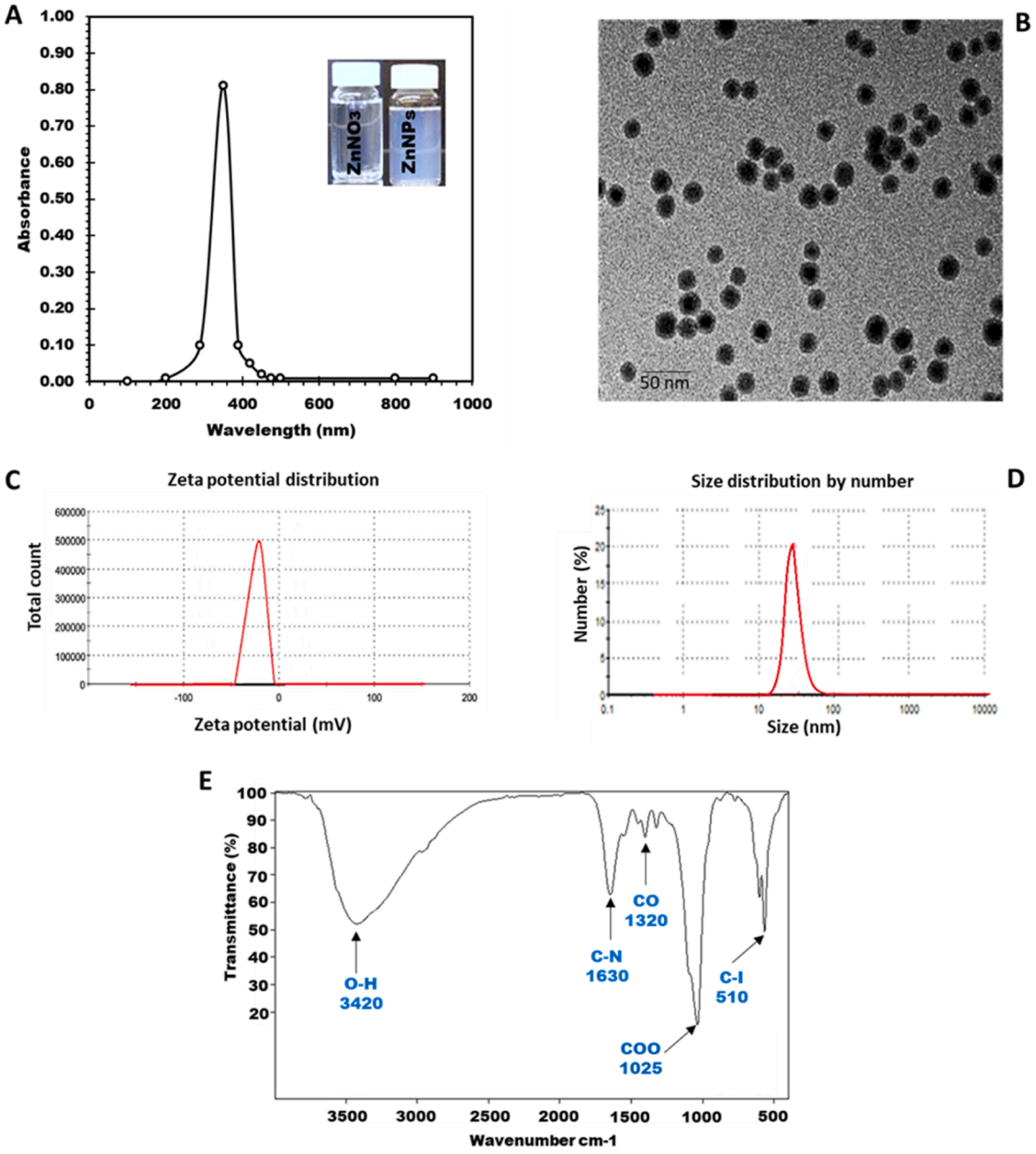

Fig. 1. Characterization of biological Lc-ZnNPs synthesized by *Lactobacillus casei* EE12. (A) absorbed UV at 365 nm, (B) spherical form, (C) -ve charge of −24.4 mV, and (D) size of Lc-ZnNPs of 41 nm. (E) FTIR analysis shows the active groups surrounding the nanoparticles.

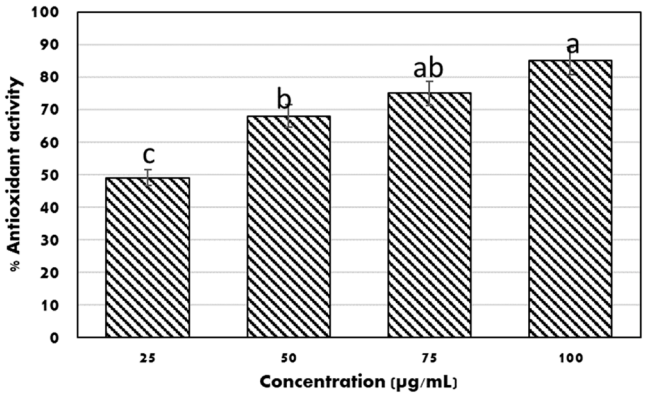

Fig. 2. The antioxidant ability of Lc-ZnNPs synthesized by *Lactobacillus casei* EE12 against DPPH free radicals. Values with an ordinary superscript letter (a, b, c) significantly differ ($P < 0.05$).

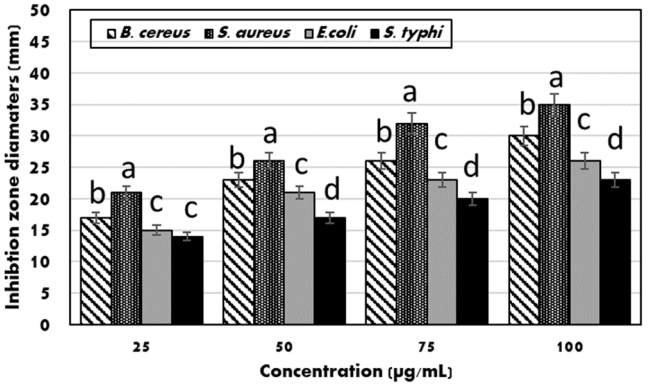

Fig. 3. Antibacterial activity of Lc-ZnNPs synthesized by *Lactobacillus casei* EE12 against broiler pathogenic bacteria *B. cereus, S. aureus, E. coli, & S. typhi*. Values with a common superscript letter (a, b, c, d) significantly differ ($P < 0.05$).

microorganisms ranged from 30 to 45 µg/mL.

3.4. Anticoccidial activity of Lc-ZnNPs

Fig. 4 shows the significant *in vitro* anticoccidial activity of Lc-ZnNPs at 25, 50, 75, and 100 µg/mL. The findings revealed that Lc-ZnNPs at various concentrations significantly decreased *Eimeria* oocysts, ranging from 75 % to 98 %. The most potent concentration was assessed to be 75 µg/mL; hence, it was selected for use in the *in vivo* experiment.

3.5. The influence of Lc-ZnNPs on OPG and lesion rate in E. tenella-infected broilers

Following experimental infection with *Eimeria tenella*, the infected control group (T4) exhibited marked clinical signs starting on day four post-infection, characterized by frequent episodes of bloody diarrhea and general weakness in the birds. Postmortem examination of recently deceased birds from the T4 group on day four post-infection (as depicted in Fig. 5) revealed severe hemorrhagic typhlitis, presenting as a "blood sausage" appearance in the caeca. By day 14 post-infection, the caeca of the T4 group showed a distinct intestinal core mixed with hemorrhagic contents. In contrast, control birds (Group C) examined at 18 days of age displayed normal intestinal morphology. These findings in the T4 group confirmed the effective induction of experimental coccidiosis, resulting in a cumulative mortality rate of 18.3 % (11 out of 60 birds) throughout the experimental period. Notably, the treatment group receiving 75 mg Lc-ZnNPs/kg diet (T5) showed a reduced incidence of bloody diarrhea,

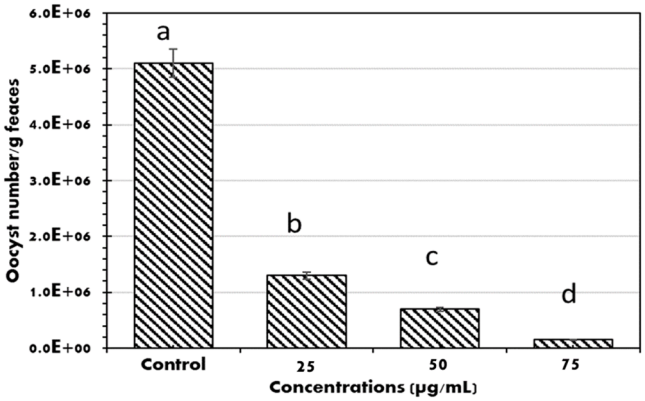

Fig. 4. Anticoccidial activity of Lc-ZnNPs fabricated by *Lactobacillus casei* EE12 against *E. tenella* oocysts. Lowercase letters denote significant differences among zinc concentrations at $P < 0.05$.

observed in only 5 out of 60 broilers (8.3 %), and no mortality was recorded in this or any other treatment group.

Oocyst shedding, quantified as oocysts per gram (OPG) of excreta on days 21 and 28 post-infection using a McMaster chamber, was significantly higher ($P < 0.05$) in the infected control group (T4) compared to all other groups. As expected, the uninfected control group (C) remained asymptomatic and did not shed any oocysts. Importantly, the Lc-ZnNPs-treated infected group (T5) demonstrated a substantial reduction in OPG compared to the infected control (T4), exhibiting a relative decrease of 67 %.

Intestinal lesion scoring, presented in Table 2, was significantly elevated in the infected control group (T4), indicative of substantial intestinal damage. Conversely, the lesion scores were markedly lower in the Lc-ZnNPs-treated infected group (T5) when compared to both the uninfected control (C) and the infected control (T4).

3.6. Growth performance

Table 3 demonstrates the impact of various Lc-ZnNPs treatments on body weight gain (BWG). The highest BWG (2.4 kg) was observed in the group receiving 75 mg Lc-ZnNPs/kg diet, representing a 10 % relative increase compared to both the un-supplemented control and the *Eimeria tenella*-infected group, which exhibited the lowest BWG (1.75 kg).

Including Lc-ZnNPs in the diet of infected broiler chickens significantly improved BWG to 1.86 kg. This enhancement can be attributed to increased feed intake (FI) and a more favorable feed conversion ratio (FCR). Specifically, the *E. tenella*-infected group (T4) exhibited a higher FCR (1.75) compared to the infected group supplemented with 75 mg Lc-ZnNPs/kg diet (T5), which showed a lower FCR of 1.52. Consequently, the supplementation of Lc-ZnNPs demonstrably enhanced the growth performance and effectively mitigated the adverse effects of *E. tenella* challenge.

3.7. Blood biochemistry

Table 4 presents the serum biochemical parameters of *Eimeria tenella*-infected broiler chickens and the mitigating effects of Lc-ZnNPs on this infection. The activity of liver enzymes was markedly elevated in the *E. tenella*-infected group, with Alanine Aminotransferase (ALT) at 3.5 U/L and Aspartate Aminotransferase (AST) at 266 U/L. However, dietary supplementation with Lc-ZnNPs in the infected group regulated these enzyme activities, resulting in relative reductions of 30 % for ALT and 43 % for AST. Uric acid levels were highest in the infected group (T4) at 5.6 mg/dL, which was reduced by 25 % in the Lc-ZnNPs-treated infected group (T5).

Dietary Lc-ZnNPs supplementation significantly improved the lipid

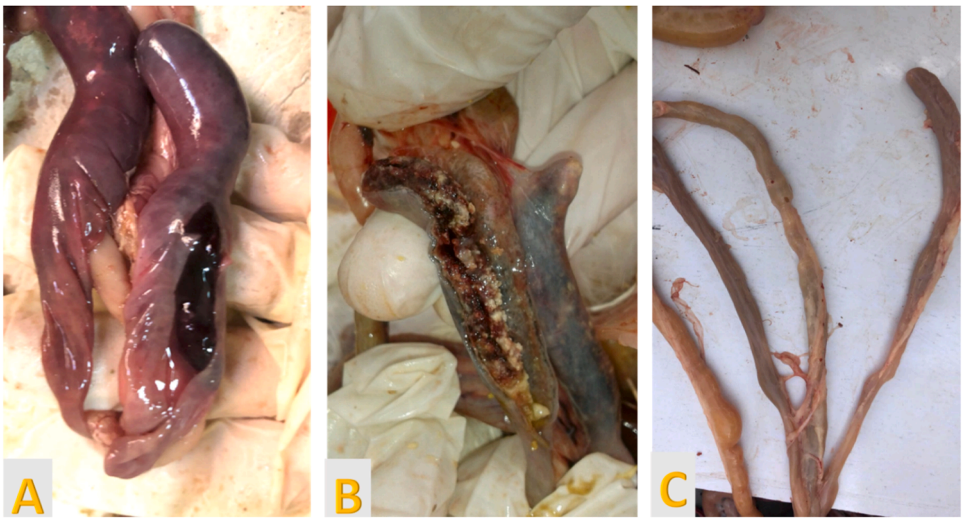

Fig. 5. (A, B) Postmortem of freshly dead birds 4 days after the experimental infection in T4 showing hemorrhagic typhlitis (blood sausage appearance), (B) at 14 days after infection in T4 showing intestinal core mixed with bloody contents, and (C) birds in the control group at 18 days old showing normal intestinal appearance.

Table 2
Impact of dietary Lc-ZnNPs treatments on OPG (x10^4) and lesion score of *Eimeria*-challenged broiler chickens.

Treatments	OPG		Lesion score
	7dpi	14 dpi	
C	0 ± 0.0	0 ± 0.0	0 ± 0.0
T1	0 ± 0.0	0 ± 0.0	0 ± 0.0
T2	0 ± 0.0	0 ± 0.0	0 ± 0.0
T3	0 ± 0.0	0 ± 0.0	0 ± 0.0
T4	9.5 ± 0.9a	3.2 ± 0.3b	2.5 ± 0.1
T5	4.2 ± 0.5a	0.11 ± 0.02b	0.7 ± 0.05

Results were presented mean ± SD. Diverse lowercase letters in the same raw elucidate the significant differences at $p < 5$ % via LSD. OPG, oocytes per gram. dpi = days post-infection.

profile in the uninfected T3 group, evidenced by decreased concentrations of total cholesterol (TC) at 95 mg/dL, low-density lipoprotein (LDL) at 18 mg/dL, and high-density lipoprotein (HDL) at 98 mg/dL. In contrast, the *E. tenella*-infected group (T4) exhibited elevated TC (139 mg/dL) and LDL (49 mg/dL) concentrations, along with a lower HDL concentration (75 mg/dL). Lc-ZnNPs supplementation in the infected group (T5) modulated these lipid parameters, resulting in TC at 105 mg/dL, LDL at 25 mg/dL, and HDL at 94.2 mg/dL.

The inclusion of Lc-ZnNPs in the broilers' diet also enhanced their immunity, demonstrated by increased concentrations of Immunoglobulin A (IgA), Immunoglobulin G (IgG), Triiodothyronine (T3), and Thyroxine (T4), showing a relative increase of 16 % compared to the infected group (T4). Furthermore, ZnNPs improved the oxidative status in the *E. tenella*-challenged birds by reducing Malondialdehyde (MDA) levels and enhancing the activities of the enzymatic antioxidant defense system, including Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPx).

3.8. Proinflammatory & proapoptotic markers

The influence of Lc- ZnNPs in the broiler diet on the related expression of the gut (OCCU & MUC-1) health markers in *E. tenella*-challenged broilers is exhibited in Fig. 6A. The gene expression of these genes was normal in the control, T1, T2, & T3 groups, contemplating intestine health. However, T4 revealed a massive upregulation in these markers genes, while adding Lc-ZnNPs downregulated these genes' expression by 50 % in group T5.

The influence of Lc-ZnNPs supply on the related expression of the kidney inflammatory (IL-6 & IL-1β) and proapoptotic (Bax & caspase-3) markers in *E. tenella*-infected broiler was expressed in Fig. 6B and C. The expression of kidney inflammatory & proapoptotic markers was normal in control, T1, T2, & T3 groups; in opposition, they significantly increased T4. The infected broilers treated with Lc-ZnNPs in T5 showed a substantial advancement in the expression of the kidney inflammatory & proapoptotic markers compared to T4.

3.9. Microbial intestinal count

Fig. 7 illustrates the impact of dietary Lc-ZnNPs supplementation on the intestinal microbial populations of broiler chickens. Across all groups receiving Lc-ZnNPs at varying concentrations (T1, T2, and T3), a statistically significant reduction was observed in the counts of total bacteria (TBC), total yeast and mold count (TYMC), *Escherichia coli*, and *Salmonella* spp.

Conversely, the population of lactic acid bacteria (LAB), specifically *Lactobacillus* spp., exhibited a notable increase in the Lc-ZnNPs-supplemented groups (T1, T2, and T3) when compared to the control

Table 3
Impact of Lc-ZnNPs dietary supply on growth performance of *Eimeria*-challenged broiler chickens.

Parameters	Age (d)	C	T1	T2	T3	T4	T5	*P*-value
LBW (g)	1	45.1 ± 0.0	45 ± 0.1	44.8 ± 0.5	45.1 ± 0.2	44.6 ± 0.2	44.5 ± 0.0	0.9
FBW (g)	35	2250 ± 0.9c	2300 ± 0.9b	2350 ± 2.2ab	2390 ± 1.0a	2200 ± 1.5e	2310 ± 2.1b	< 0.001
BWG (g)	1–35	1800 ± 0.2d	1850 ± 1.1c	1900 ± 1.6b	1940 ± 1.0a	1750.4 ± 0.9e	1860 ± 1.9c	< 0.001
FI (g)	1–35	3520 ± 2.3d	3590 ± 2.1c	3600 ± 1.4b	3660 ± 2.1a	3410 ± 1.2e	3550 ± 0.9cd	<0.001
FCR	1–35	1.62 ± 0.1c	1.66 ± 0.2c	1.71 ± 0.5b	1.75 ± 0.1a	1.52 ± 0.6e	1.6 ± 0.2c	< 0.05
GR	1–35	194.2 ± 1.2b	195.6 ± 1.1b	196.3 ± 1.2b	197.3 ± 1.3a	186 ± 2.1e	192 ± 3.2c	< 0.05
PI	1–35	121.3 ± 1.0d	132.5 ± 0.6c	142.6 ± 1.0b	149.8 ± 1.5a	138 ± 2.8e	141.3 ± 2.0b	< 0.001

Results are displayed mean ± SD. Diverse lowercase letters in the same raw elucidate the significant differences at $p < 5$ % via LSD.

Table 4
Impact of dietary Lc-ZnNPs supply on renal and hepatic function, lipid profile and immunity parameters of *Eimeria*-challenged broiler chickens.

Serum parameters	Experimental groups/ dietary ZnNPs (mg/kg)						*p* value
	C	T1	T2	T3	T4	T5	
				Biomarkers of Liver and kidney			
Aspartate Transaminase (U/L)	252 ± 3.1b	222 ± 2.2c	210 ± 3.0d	175 ± 1.9f	266 ± 3.2a	187 ± 1.2e	<0.0001
Alanine aminotransferase (U/L)	3.1 ± 0.2ab	2.8 ± 0.2b	2.1 ± 0.2c	1.8 ± 0.1d	3.5 ± 0.1a	2.0 ± 0.1d	<0.00001
Creatinine (mg/dl)	0.31 ± 0.00	0.33 ± 0.03	0.34 ± 0.07	0.27 ± 0.01	0.37 ± 0.06	0.33 ± 0.05	0.99
Uric acid (mg/dl)	5.5 ± 0.4ab	4.77 ± 0.7b	4.41 ± 0.1c	3.7 ± 0.2d	5.6 ± 0.5a	4.2 ± 0.5c	0.0025
				Lipid Markers			
TCholesterol (mg/dl)	132 ± 1.2ab	127 ± 1.5b	122 ± 1.9b	95 ± 2.3d	139 ± 1.8a	105 ± 1.3c	<0.00001
LDLipoprotein (mg/dl)	45 ± 0.1ab	33 ± 0.3b	27 ± 0.2c	18 ± 0.5d	49 ± 0.7a	25 ± 0.1c	<0.00001
HDLipoprotein (mg/dl)	92 ± 1.6b	94 ± 1.8ab	95 ± 2.7ab	98 ± 1.3a	75 ± 1.2c	94.2 ± 1.1ab	<0.00001
Gut fat	1.3 ± 0.2a	1.0 ± 0.3ab	0.88 ± 0.05b	0.71 ± 0.0c	1.41 ± 0.8a	0.84 ± 0.03b	<0.00001
				Immunity			
IgG (mg/dl)	953.3 ± 4.2e	1050 ± 5.1d	1071 ± 1.6c	1095 ± 3.2a	941 ± 3.6f	1088 ± 3.5b	0.001
IgA (mg/dl)	175.8 ± 0.9e	187.2 ± 1.1d	190.3 ± 1.4c	203.6 ± 1.2a	171 ± 2.3e	198.2 ± 1.7b	0.001
T3 (ng/dl)	2.35 ± 0.0	2.32 ± 0.1	2.31 ± 0.2	2.30 ± 0.0	2.33 ± 0.1	2.35 ± 0.1	0.9
T4 (ng/dl)	135.1 ± 1.1	134.3 ± 1.9	134 ± 0.9	136 ± 0.8	133 ± 1.5	131.6 ± 1.1	0.5
				Antioxidant status			
MDA (nmol/ml)	215.2 ± 5.6bc	213 ± 3.2bc	210 ± 3.9bc	198 ± 2.2d	489 ± 9.2a	222 ± 3.6b	<0.0001
SOD (U/ml)	95.3 ± 3.6ab	95.6 ± 1.2ab	96.4 ± 1.9ab	98.6 ± 2.1a	49 ± 2.1c	85 ± 2.1b	<0.0001
CAT (U/ml)	193.6 ± 3.3b	195 ± 1.9b	198 ± 2.3ab	199.2 ± 2.5a	105 ± 3.1d	181 ± 2.5c	<0.0001
GPx (U /ml)	2.23 ± 0.1b	2.25 ± 0.2b	2.27 ± 0.3b	2.31 ± 0.1a	1.0 ± 0.1c	2.0 ± 0.1bc	<0.0001

Results were exhibited mean ± SD, Significant differences in the same raw were indicated by different (a-e) at $P < 0.05$ via LSD.

group (C).

The Eimeria-challenged group (T4) demonstrated elevated counts of TBC (7.2 CFU/g), TYMC (7.0 CFU/g), and *Salmonella* spp. (3.7 CFU/g) compared to the unchallenged control group (TBC: 3.8 CFU/g, TYMC: 2.6 CFU/g, *Salmonella*: 3.2 CFU/g). However, the Eimeria-challenged group receiving Lc-ZnNPs supplementation (T5) showed a significant decrease in these detrimental microbial populations (TBC, TYMC, and *Salmonella* spp.) and a concurrent enhancement in the LAB count when compared to both the Eimeria-challenged control group (T4) and the unchallenged control group (C). This indicates a potential prebiotic-like effect of Lc-ZnNPs in modulating the gut microbiota, particularly in coccidial challenge.

3.10. Histopathology

Fig. 8 shows different intestinal sections of non-infected, Lc-ZnNPs-treated, and *E. tenella*-infected chicks. The histology of the control, T1, T2, and T3 groups revealed typical structures of the intestines. In contrast, the *E. tenella*-infected group (T4) exhibited exfoliation of the gastrointestinal lining and a significant infection of the intestinal tissues (black arrows) with parasite schizonts when Lc-ZnNPs were added as an anticoccidial agent. Fig. 8F suggests a reduction in parasites within the intestinal lining and the occurrence of very few parasites.

4. Discussion

Zinc (Zn) is a crucial trace mineral for both mammals and birds, playing diverse roles in regulating nutrient metabolism, modulating the immune system, controlling appetite, scavenging free radicals (FR), and regulating transcription factors [71]. It is also essential for the synthesis and breakdown of macromolecules and acts as a vital component of numerous enzymes [72]. The National Research Council (NRC) recommends 40 mg/kg of dietary zinc for broiler chickens [73].

However, current NRC recommendations for trace minerals in broiler diets are based on research conducted with older, slower-growing broiler breeds [74] and may not be optimal for the faster growth rates of modern commercial strains [75]. To optimize performance, broiler producers often supplement inorganic trace minerals, such as oxides or sulfates, at levels exceeding NRC recommendations [76]. Consequently, the mineral premix used in this study contained 75 mg/kg of inorganic zinc oxide, serving as a necessary nutritional component in the baseline diet of the broiler chicks [77].

Coccidiosis is a widespread and economically significant parasitic disease in poultry caused by protozoan parasites of the genus *Eimeria*. These parasites infect the intestinal tract of chickens, leading to malabsorption of nutrients, diarrhea (often bloody), reduced growth rates, increased susceptibility to other diseases, and potentially high mortality rates, particularly in young birds. Control strategies have traditionally relied on anticoccidial drugs, but the emergence of drug-resistant *Eimeria* strains necessitates the exploration of alternative preventative and therapeutic approaches.

In the current study, the *E. tenella*-infected control group exhibited a mortality rate of 18.3 % (11/60) and notable bloody diarrhea. In contrast, the infected group receiving dietary Lc-ZnNPs (T5) showed a reduced incidence of bloody diarrhea (8.3 %, 5/60 broilers) and no mortality. These findings align with previous research by Attia et al. [2], who reported a 15 % mortality rate in experimentally challenged broilers. This study supplemented the experimentally challenged chickens with 75 mg/kg of ZnNPs.

Numerous studies suggest that zinc can enhance broiler feed intake [78]. For example, broilers fed 2.5 mg/kg ZnNPs showed a significant increase in feed intake compared to the control group ($P < 0.05$) [79]. Similarly, ZnNPs supplementation at 10, 20, and 40 mg/kg significantly increased feed intake ($P < 0.001$) compared to control birds [80]. Moreover, adding 120 mg/kg of ZnNPs to the broiler diet significantly increased feed intake compared to the control ($P < 0.05$) [80]. A significant increase in feed intake was also observed in broilers supplemented with 50 mg/kg zinc methionine and ZnNPs ($P < 0.05$) [81]. However, other researches indicated that ZnNPs supplementation at levels ranging from 5 to 80 mg/kg did not affect the feed intake of broilers during their lifetime [82]. Regarding body weight gain, several studies reported a significant increase in broilers fed 2.5 mg/kg [79], 40 mg/kg [83], or 40, 80, and 160 mg/kg ZnNPs [84] compared to the control ($P < 0.05$, $P < 0.001$, & $P < 0.001$, respectively).

Several authors have investigated the influence of diet, including zinc, on the blood biochemistry of broiler chickens. Research has shown lower serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in broilers and laying hens fed zinc-supplemented diets compared to controls. Mohammed et al. [85] reported that ALT and AST serum levels were significantly decreased at 35

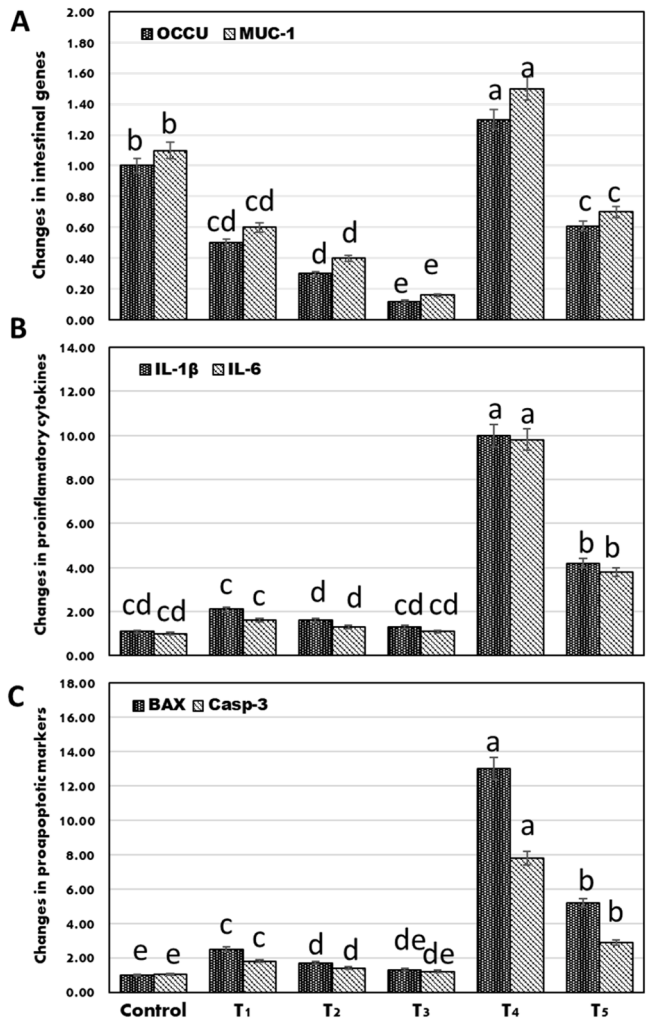

Fig. 6. The effect of Lc-ZnNPs on the relative expression of (A) intestine health markers (MUC-1& OCCU), (B) proinflammatory cytokines (IL-1β, IL-6), & (C) proapoptotic markers (Casp-3 & BAX) in *E. tenella*-infected broiler chickens. Values with (a, b, c, d, e) significantly differ ($P < 0.05$).

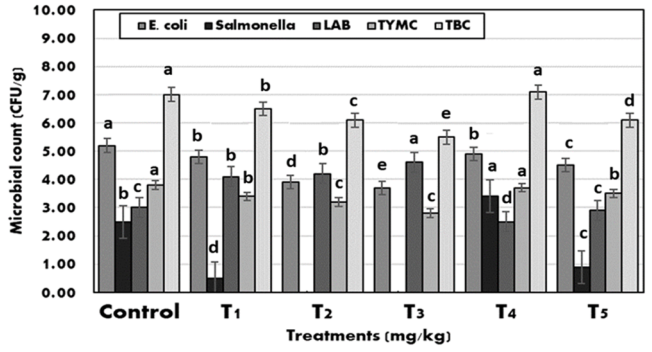

Fig. 7. The effect of dietary Lc-ZnNPs on intestinal microbiota (*E. coli, Salmonella*, LAB, TYMC, and TBC) counts in *E. tenella*-challenged birds. a-e lowercase letters above columns indicate significant differences between bacterial count in treatments.

days of age in broilers receiving ZnNPs (60, 80, 100, and 120 mg/L) in drinking water compared to a control group ($P < 0.05$). Fawaz et al. [86] observed a linear decrease in ALT and AST levels in mice fed a diet containing ZnNPs at 20, 40, and 60 mg/kg compared to the control ($P < 0.001$) [87]. Similarly, AST and ALT activities in the serum were significantly reduced in animals fed a diet containing 30 mg/kg of ZnNPs compared to the control ($P < 0.05$) [88].

This study aimed to evaluate the effectiveness of ZnNPs as a preventative strategy against coccidiosis. The findings in the *E. tenella*-infected control group demonstrated effective disease induction, with a mortality rate of 11.9 % and notable bloody diarrhea, which, according to Dkhil et al. [89], can indicate coccidial infection.

Zinc is known to significantly impact coccidiosis, leading to an increase in oxidative stress (OS) through the release of reactive oxygen species (ROS) and impaired nutrient absorption [90]. Elevated levels of Malondialdehyde (MDA) and reduced levels of total antioxidant activity (AOA) observed during the fourth week after challenge are indicative of OS contributing to the development of coccidiosis [91]. The scavenging activity of ZnNPs was evidenced by a significant increase in antioxidant activity in treated animals compared to the control group. The treated groups effectively suppressed OS during the fourth-week post-challenge. Due to its role as a ROS scavenger, zinc in ZnNPs maintained oxidative stability in chicks infected with coccidiosis [92].

ZnNPs inhibited the reproduction of *Eimeria*, as indicated by the reduction in lesion scores and oocyst shedding. Do Carmo Neto et al. [93] reported a significant decrease in *Eimeria* oocysts in the feces of mice administered ZnO—NPs. Furthermore, in rabbits infected with *E. steidae*, ZnNPs administration resulted in no detectable presence of the protozoal phase in the liver [94].

The growth performance traits of *Eimeria*-infected birds in this study were the lowest, consistent with prior research [95]. This growth decline is likely attributable to intestinal damage and reduced villi length [96]. The groups infected but treated with ZnNPs exhibited enhanced growth performance, likely due to reduced intestinal damage. ZnNPs are considered more efficient than conventional zinc sources in improving feed utilization and promoting growth due to zinc's involvement in numerous metabolic enzymatic reactions [88]. Moreover, they enhance growth by increasing intestinal villi length and crypt depth, leading to improved nutrient absorption.

The study findings revealed a significant occurrence of bloody droppings and the highest quantity of oocysts excreted in the feces of the *Eimeria*-infected broilers. Bloody diarrhea results from the infiltration of sporozoites into the cecal epithelium. The subsequent developmental phases of the protozoan, including schizogony, ultimately damage intestinal blood vessels, causing hemorrhages [97,98]. This study found that 60 mg/kg ZnNPs (equivalent to 71 mg/kg of Zn) significantly decreased the oocyst quantity of *E. tenella* in feces, the severity of cecal lesions, and the anticoccidial index. Similarly, Anah et al. [99] found that zinc oxide nanoparticles (ZnONPs) demonstrated anticoccidial activity during the first week of *E. tenella* infection, with 60 mg/kg being the most effective in reducing oocyst shedding compared to both infected controls and the amprolium treatment group. This highest dose also resulted in milder clinical signs and a low mortality rate of 0.8 %. During the second week, oocyst excretion and mortality declined across all ZnONPs -treated groups, with the 60 mg/kg ZnONPs group exhibiting the lowest oocyst counts and complete recovery by the end of the second week, showing superior efficacy compared to other treatments. A recent study investigated the potential of organic zinc (OZn) and methionine (Met) supplementation to combat *Eimeria tenella* infection in broilers. Compared to uninfected controls, broilers challenged with *E. tenella* showed significantly higher mortality, lesion scores, and oocyst counts. However, 50 mg/kg OZn and 0.56 % Met significantly reduced these adverse effects, improving growth performance and cecal morphology. This combined treatment proved more effective than OZn or Met alone, demonstrating a synergistic anticoccidial effect and mitigating the adverse impacts of coccidiosis on broiler growth [100]. This effect of ZnNPs may be attributed to the inhibition of parasite population within intestinal cells before infecting and releasing infective oocysts, subsequently reducing OPG in *E. tenella*-infected birds [101]. Bami et al. [102] reported that supplementing ZnNPs at 40 mg/kg is a significant feed additive for birds, with beneficial impacts on intestinal morphology in a

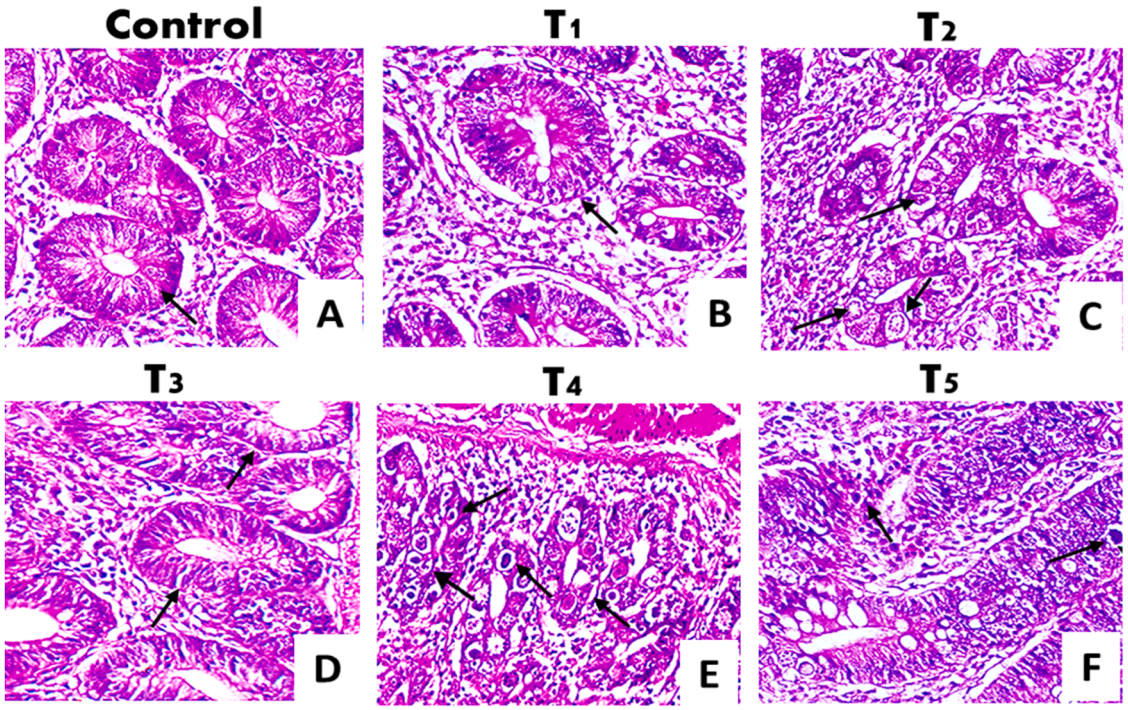

Fig. 8. The histopathology of intestinal tissues was examined in different groups: (A) control, (B) T1, (C) T2, (D) T3, (E) T4, (F) T5. Control, T1, T2, and T3, (arrows indicate typical structures in the intestines), T4 (The loss of the intestinal lining indicates a gastrointestinal severe tract infection with parasite schizonts), & T5 (The arrow shows a decrease in parasites inside the gut wall, with only a small number remaining). The sample was stained using H&E, and the scale bar represents a length of 100 μm at a magnification of 100X.

comprehensive experiment. In all parts of the small intestine of ZnNPs-supplemented birds, a significant improvement in total goblet cell count, villus height, villus surface area, and an elevated villus height:crypt depth ratio was observed. Bahrampour et al. [103] explained that increased villus height could be due to enhanced bioavailability of zinc nanoparticles, thereby preserving epithelial barrier integrity and activity. Adequate villus height in the gut is crucial for the acidic mucin to withstand microbial destruction, resulting in less cellular degradation [104,105]. Similarly, crypt development is necessary to improve the villi maturation rate and renewal. Birds receiving ZnNPs exhibited substantial development improvements, indicated by lower scores for intestinal injuries, suggesting enhanced intestinal health and regulation of gut microbes [105]. Additionally, ZnNPs are more potent than regular zinc oxide in improving feed utilization and development, likely because zinc is a vital element for several metabolic enzymes. Consequently, ZnNPs may provide a supply function that improves development.

The current data demonstrated a significant increase in hepatic enzymes in the serum of *Eimeria*-challenged chickens. The rise in enzyme activity is a consequence of disruption to the cecal cell wall, inflammation, and significant blood loss. ZnNPs decreased the activity of these enzymes. Furthermore, it was noted that ALT activity increased after the *E. tenella* challenge, whereas AST levels remained unaffected by the disease. This discrepancy in findings may be due to fluctuations in intestinal inflammation, gut injury, and hemorrhages [99].

The antioxidant activity evaluation revealed a marked decrease in the levels of Catalase (CAT) and Superoxide Dismutase (SOD), indicating the occurrence of oxidative damage induced by *Eimeria* challenge. The treated birds showed high SOD and CAT levels, which aligns with the results of prior findings that registered a marked rise in SOD and CAT levels due to ZnNPs supplementation [84]. Our data showed that the scavenging activities of ZnNPs were improved, consistent with the results of prior studies [84], which stated that supplementing ZnNPs in the feed boosts the activities of SOD and CAT in the blood; however, no statistically significant alteration in antioxidant activity was observed when broilers were given 15–60 mg/kg nano-zinc [88]. Likewise, an investigation has suggested that ZnNPs at a 60 mg/kg dose display beneficial Cu-Zn-SOD activities; ZnNPs have a remarkable antioxidant impact due to the presence of Zn, an essential element of SOD, and this enzyme efficiently combats OS by rapidly removing superoxide free radicals. ZnNPs demonstrate antioxidant effects by competing with iron (Fe) and copper (Cu) for binding to specific sites on cell membranes. This competition decreases the creation of free radicals, hence reducing OS.

Furthermore, it is recommended that zinc improves the production of metallothionein, a crucial protein in neutralizing damaging free radicals [106]. Zinc also initiates antioxidant enzymes and proteins, such as Glutathione Peroxidase (GPx) and CAT [107]. The *Eimeria*-infected birds showed a substantial rise in proinflammatory cytokines, as implied by the current work.

In the *Eimeria*-infected group, proinflammatory cytokines substantially increased in the inflamed gut due to their attraction towards the site of inflammation. Supplementing ZnNPs diminished the concentrations of proinflammatory cytokines, particularly Interleukin-2 (IL-2) and Tumor Necrosis Factor-alpha (TNF-α). This work concurs with prior data [84] that ZnNPs reduce blood IL-2 and TNF-α levels. This anti-inflammatory impact of zinc is likely due to the triggering of the Nuclear factor erythroid 2-related factor 2/Heme oxygenase-1 (Nrf2/HO-1) signaling pathway [108]. Activation of the Nrf2/HO-1 pathway lessens the formation of proinflammatory cytokines, promotes the formation of immunoglobulins, and effectively halts the inflammatory reactions mediated by Toll-like receptor 4 (TLR4) [109,110], thus suggesting the anti-*Eimeria* impact of ZnNPs and a growth performance enhancement.

The control of *Eimeria*-induced coccidiosis in poultry has seen significant advancements in recent years, driven by the urgent need to address drug resistance and improve sustainable farming practices. Recent studies highlight zinc oxide nanoparticles (ZnO—NPs) as a promising intervention, with research demonstrating their efficacy at 40–60 mg/kg in reducing *E. tenella* oocyst shedding and intestinal lesions through oxidative stress-mediated parasite damage [47,111].

Beyond ZnO—NPs, silver nanoparticles (AgNPs) and selenium nanoparticles (SeNPs) have emerged as potent alternatives, with AgNPs inhibiting sporulation by 90 % [112] and SeNPs improving immune responses and antioxidant capacity [113]. Vaccine development has also progressed, with next-generation subunit vaccines targeting *Eimeria* antigens (*e.g.*, MIC3) showing improved mucosal immunity [21], while CRISPR-edited live attenuated vaccines offer safer and more immunogenic profiles [114]. However, drug resistance remains a critical issue, with 2024 surveys reporting over 50 % resistance to toltrazuril and diclazuril in commercial flocks, prompting the adoption of shuttle programs alternating ionophores and synthetics with prolonging drug efficacy [115]. Simultaneously, gut microbiota modulation has gained traction, as probiotics (*e.g., Bacillus subtilis*) strengthen intestinal barriers and reduce *Eimeria* pathogenicity and prebiotics (FOS/MOS) lower oocyst shedding by 20–30 % [5]. Adding nanoparticles or heterogeneous nanocomposites also influences the microbiota [116–118]. Technological innovations like AI-based early detection systems now enable pre-symptomatic identification of coccidiosis through behavioral monitoring [119], and automated litter management minimizes environmental oocyst loads. Looking ahead, the integration of nanoparticles, CRISPR-based vaccines, and precision farming is poised to redefine coccidiosis management. Future research must prioritize field validation of mRNA vaccines, optimization of nanoparticle delivery systems, and global surveillance of drug resistance to ensure sustainable control. The findings collectively underscore a shift toward multidisciplinary strategies that balance efficacy, animal welfare, and environmental safety in the fight against avian coccidiosis.

5. Conclusions

Lc-ZnNPs show significant promise as an alternative treatment for coccidiosis in broiler chickens. Research on *Eimeria tenella*-infected birds revealed that Lc-ZnNPs supplementation effectively reduced oocyst shedding, improved growth performance, and limited tissue destruction. The beneficial effects of Lc-ZnNPs also extend to their anti-inflammatory and antioxidant properties, demonstrated by increased SOD and CAT activity and decreased pro-inflammatory cytokine levels.

Abbreviations

The following abbreviations are used in this manuscript:

Lc-ZnNPs	*Lactobacillus casei*-Zinc nanoparticles
MHA	Muller-Hinton agar
FI	Feed intake
BWG	Body weight gain
FCR	Feed conversion ratio
GR	Growth rate
PI	Performance index
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CK	Creatin kinase
MDA	Malondialdehyde
SOD	Superoxide dismutase
CAT	Catalase
GPx	Glutathione peroxidase
TBC	Total Bacteria Count
TYMC	Total Combined Yeasts/Molds Count
DPPH	2,2-diphenyl-1-picrylhydrazyl
IZDs	Inhibition zone diameters
IL-2	Interleukin-2
TNF-α	Tumor necrosis factor-alpha
FRs	free radicals

CRediT authorship contribution statement

Felwa Abdullah Thagfan: Supervision, Methodology, Conceptualization. **Narjes Baazaoui:** Resources, Data curation. **Soha A. Alamoudi:** Methodology, Software, Data curation. **Mina A. Almayouf:** Writing – original draft, Software. **Hayat S. Al-Rashidi:** Visualization, Methodology. **Samar Ahmad Khan:** Methodology, Investigation. **Amal M. Alzahrani:** Visualization, Methodology, Investigation. **Mario Nicotra:** Resources, Data curation. **Carlotta Marini:** Writing – review & editing, Investigation. **Alessandro Di Cerbo:** Writing – review & editing, Writing – original draft, Resources, Conceptualization. **Mohamed T. El-Saadony:** Writing – review & editing, Writing – original draft, Methodology, Investigation.

Declaration of competing interest

Authors declare no conflict of interests.

Data availability statement

The data presented in this study are available on request from the corresponding author.

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