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Evaluation of antibacterial activity of zinc oxide nanoparticles against avian mycoplasmosis with assessment of its impact on broiler chickens' performance and health

Mohamed Shakal[1], Fady S. Youssef[2], Gehad G. Mohamed[3,4], Sameh H. Ismail[5], Heba M. Salem[1*]

[1]*Department of Poultry Diseases, Faculty of Veterinary Medicine, Cairo University, 12211, Giza, Egypt.*
[2]*Pharmacology Department, Faculty of Veterinary Medicine, Cairo University, 12211 Giza, Egypt.*
[3]*Chemistry Department, Faculty of Science, Cairo University, 12613 Giza, Egypt.*
[4]*Nanoscience Department, Basic and Applied Sciences Institute, Egypt-Japan University of Science and Technology, New Borg El Arab, Alexandria, 21934, Egypt.*
[5]*Nanotechnology for postgraduate studies - Cairo University- Sheikh Zayed Branch Campus, Sheikh Zayed City, Giza PO 12588, Egypt.*

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***Correspondence:**

Corresponding author: Heba M. Salem
E-mail address: dr.hebasalem@cu.edu.eg

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ABSTRACT

Mycoplasmosis is a main threat to the poultry industry. Recently, *Mycoplasma* developed resistance against most macrolides therefore, this study aimed to *in vitro* and *in vivo* evaluation of the antimicrobial activity of zinc oxide nanoparticles (ZnO-NPs) against *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS). *In vitro* studies were adopted to estimate the minimum inhibitory concentration (MIC) of ZnO-NPs against MG and MS. For *in vivo* studies, 189 birds were allotted into 9 groups including 21 birds/group with triplicates, 7 birds each as follow: G1: challenged with MS; G2: challenged with MS and treated with 1% ZnO-NPs; G3:; challenged with MS and treated with 0.5% ZnO-NPs G4: challenged with MS and treated with difloxacin; G5: challenged with MG; G6: challenged with MG and treated with 1% ZnO-NPs; G7: challenged with MG and treated with 0.5% ZnO-NPs; G8: challenged with MG and treated with Difloxacin and G9: control negative. In conclusion, ZnO-NPs revealed *in vitro* anti-*Mycoplasma*l activity, the addition of 1% ZnO-NPs in the drinking water of the birds was a very effective medication for controlling MG & MS infections in broiler and treated birds revealed better FCR, a significant reduction in the severity of clinical signs and lesion score, a significant improvement in antioxidant status (Catalase, MDA & GSH), enhancement of hepatic (ALT & AST) & renal function (Creatinine & Urea) and a significant improvement in lipid profile (cholesterol & triglycerides). The usage of 1% ZnO-NPs is recommended as a safe effective treatment against avian mycoplasmosis in broiler chickens.

Introduction

Avian mycoplasmosis is a major hazard revealing severe economic losses in poultry industry all over the world (El-Naggar *et al*., 2022; Marouf *et al*., 2022a). *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are the most abundant types attacking avian species resulting in financial losses in terms of decreased final weight, lowered egg production, and hatchability, increased embryonic mortality, increased carcass condemnation, high prophylaxis, and treatment costs (Yadav *et al*., 2022; Limpavithayakul *et al*., 2023; Wang *et al*., 2023). Given that Mycoplasmas are difficult to isolate, and MIC assessments take a long time to produce results, most of the antimicrobial medications given to animals are typically empirical instead of being recommended based on actual susceptibility data (Gigueré, 2013; Ferguson-Noel *et al*., 2020; Qoraa *et al*., 2023a,b). Since a long time ago, chicken flocks have routinely utilized macrolides to treat respiratory conditions linked to MG and MS (Awad *et al*., 2022). Due to the continuous usage of macrolides for either the prophylaxis or the treatment of avian mycoplasmosis, recently some *Mycoplasma* strains showed resistance to macrolides (Emam *et al*., 2020). As a result, monitoring MICs in Mycoplasmas is therefore still essential for identifying anti-Mycoplasma drug resistance development brought on by incorrect antimicrobial medication use (Bottinelli *et al*., 2022). Therefore, to overcome *Mycoplasma* resistance new safe alternative approaches should be applied (Abd El-Hack *et al*., 2022; Chen *et al*., 2023; Wang *et al*., 2023). One of the routes to nanotechnology is the field of nanoparticles (NPs), which is connected to nanoscale materials with extremely small particle sizes ranging from 1 to 100 nm and because of their incredibly small size and high surface area to volume ratio, NPs have unique features that significantly differ from those of their bulk counterparts (Abd El-Ghany *et al*., 2021). Zinc oxide nanoparticles have attracted a lot of attention lately owing to their distinctive characteristics. Additionally, research has indicated that zinc is a crucial mineral for living creatures (Mohd Yusof *et al*., 2019; Lail *et al*., 2023). Zinc oxide nanoparticles have a wide range of antimicrobial activity against most pathogens, in this way, adding ZnO-NPs to poultry can enhance performance and growth while acting as a different antibacterial agent to prevent disease (Mohd Yusof *et al*., 2021; Yusof *et al*., 2023). Also, the antioxidant action of zinc and its involvement in the antioxidant defense system are two of its most important characteristics (Powell, 2000). Additionally, zinc is a component of numerous proteins involved in immunological defence mechanisms, hormone secretion routes, and intermediate metabolism (Sunder *et al*., 2008). Therefore, this work was designed to *in vitro* and *in vivo* evaluation the antimicrobial, and antioxidant status of ZnO-NPs against MG and MS as well as study its effect on the performance, liver and kidney functions and blood indices of the broiler chickens.

Materials and methods

Ethical approval

The study protocol was approved by The Institutional Animal Care and Use Committee (Vet. CU. IACUC) of the Faculty of Veterinary Medicine, Cairo University, Egypt with code "Vet CU 01122022558".

Zinc oxide nanoparticle preparation and characterization

Synthesis method

ZnO NPs were synthesized using ultrasonic irradiation. In a typical

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procedure, zinc nitrate (Zn (NO_3), Loba Chemie, India) was used as a source material (Ismail *et al*., 2021). ZnO nanoparticles were synthesized by dissolving 2g zinc nitrate in 200 mL of deionized water. Subsequently, the dissolved zinc nitrate solution was ultrasonically irradiated at 55°C for 25 min using a Hielscher UP400S (400 W, 60 kHz, Germany) with an amplitude of 91% and a cycle of 0.73. Then under the action of ultrasonic irradiation, the ammonia solution is added drop by drop into the zinc nitrate solution until sol-gel formation about pH 11. Gel calcination in muffle at 700°C for 3 hours.

Characterization of the prepared Zno-NPs

The synthesized ZnO-NPs was analyzed by X-ray diffractometer (XRD, D8-Discover, USA) to determine the chemical structure and phase of crystal under condition of 1600W speed scan 0.05. Scanning Electron Microscope (SEM, JSM-6701F Plus, JEOL, USA), and the Transmission Electron A Microscope (JEOL, TEM-2100, USA) operated at a voltage of 25 kV was used to study the surface morphology, shape, and size of ZnO-NPs. When examining the samples by SEM, grid was prepared to support the NPs by sputtering with gold, but first ZnO-NPs was diluted (1:10) with doubled deionized distilled water and sonicated to more dispersed of ZnO-NPs by an ultrasonic cleaner (Elma, Germany) for 30 min. Then, a few drops of ZnO-NPs were put on the grids and allowed to dry at 60°C before SEM studies.

Mycoplasma gallisepticum and Mycoplasma synoviae strains

The MG and MS strain that have been used in this study were previously identified by Marouf *et al*. (2020). The infective dose was obtained from 48 h incubated strains on PPLO broth, and they were adjusted to 0.5 McFarland, resulting in a suspension with around 10^8 CFU/ml and then at one day old birds were infected via nasal drops (drop in each opening) with MG and via foot pad injection with 0.5 ml MS suspension. The birds were re-infected again with MG & MS at day 10 of birds age with the same dose and route.

Difloxacin (Acto pharma) was used according to the manufacture guide for the treatment of both MG and MS in the drinking water for 5 successive days as a chemical therapeutic control as recommended by Mohamed *et al*. (2022).

In vitro antimicrobial evaluation

Determination of minimum inhibitory concentration (MIC)

MG and MS isolates were treated with a range of ZnO-NPs and Difloxacin concentrations (1000 to 0.48 µg/ml). The experiment was designed to determine the lowest concentration that completely prevents MG and MS growth, as described by Emam *et al*. (2020) with some modification.

Determination of MIC

MIC of ZnO-NPs and difloxacin of MG and MS was adopted using the microdilution method (Andrews, 2001). In summary, 48 h broth cultures of bacteria were suspended in PPLO broth with turbidity adjusted to 0.5 McFarland, resulting in a suspension with around 10^8 CFU/ml (Andrews, 2001). To evaluate the MIC, 50 µl of PPLO Broth culture was poured into 12 wells of a 96-well microtiter plate. In the first well, we added 50 µl of the ZnO-NPs or difloxacin stock solution. A 2-fold serial dilution was adopted to obtain various ZnO-NPs or difloxacin concentration in each well (1000, 500, 250, 125, 62.5, 31.25, 15, 62, 7.81, 3.9, 1.95, 0.97, & 0.48 µg/ml). Then, 50 µl of the microbial suspension was added to each well. The microplate was then incubated under microaerobic condition at 37°C for 72 h, and the ZnO-NPs or difloxacin concentration in the well without visible growth of the bacterial cells was considered the MIC. A positive control includes PPLO broth medium with evaluated bacterial concentrations and a negative control contained only inoculated broth. All the measures were triplicate to confirm its value for the tested bacteria.

The MIC was defined as the least concentration of ZnO-NPs or difloxacin that visually inhibited the bacterial growth after 72 h of incubation (Andrews, 2001). The MIC was reported by observing the visual turbidity of the tubes before and after incubation. All the measures were triplicate to confirm its value for the tested bacteria.

In vivo antimicrobial evaluation

Experimental Design

One hundred ninety-five, one-day-old healthy broiler chicks were used for this experiment, at birds' arrival five birds were ethically slaughtered and pooled samples were collected from trachea, lungs, ais sacs, and synovial fluid and samples were subjected to routine bacteriological isolation to ensure the absence of MG & MS natural infections. The rest number of the birds kept in a clean house on deep litter system and supplied with antibiotic and antiparasitic-free balanced formulated commercial ration, feed and drinking water will be supplied *ad libitum* for 35 days. The birds administered routine vaccines against AI, ND, IB, and IBD viruses at the recommended time.

One hundred and eighty-nine birds were allocated into 9 groups including 21 birds/group and each group was subdivided into 3 replicates including 7 birds each as follow: G1: control positive challenged with MS; G2: challenged with MS and treated with 1% ZnO-NPs; G3:; challenged with MS and treated with 0.5% ZnO-NPs G4: challenged with MS and treated with Difloxacin; G5: control positive challenged with MG; G6: challenged with MG and treated with 1% ZnO-NPs; G7: challenged with MG and treated with 0.5% ZnO-NPs; G8: challenged with MG and treated with Difloxacin and G9: kept as control negative untreated and un-challenged birds. All treatments in different groups were started at 14 days of age and continued for 5 successive days.

Evaluation of Bird Performance

Clinical signs, Mortalities, Body Weight (BW) and Feed Conversion Rate (FCR)

During the experimental period the observation of clinical signs and mortalities was applied daily, feed consumption and body weight were evaluated for all birds weekly.

Blood samples were ethically collected from wing vein on plain tubes to separate serum from 3 birds/replicate at 14 days old (before the treatments) and at 20 days old (after the treatments). At day 20 of age, 3 randomly selected birds were ethically slaughtered from each replicate to record macroscopic lesion score. The respiratory manifestations were individually observed in chickens and the air sacs postmortem lesions of MG of dead and slaughtered birds during and after medications were reported and scored according to Kempf *et al*. (1998) while the clinical signs and postmortem lesions due to MS were reported and scored according to Kleven *et al*. (1972, 1975).

Blood chemistry

Antioxidant parameters (Catalase, MDA, GSH)

Catalase activity and reduced glutathione (GSH) level were determined using kits of Biodiagnostic Company (Dokki, Egypt according to the method described by Aebi (1984) and Beutler *et al*. (1963), respectively.

Thiobarbituric acid (TBA) react with malodialdehyde (MDA) in acidic

medium at temperature of 95˚C for 30 min to form thiobarbituric acid reactive product, the absorbance of the resultant pink product was measured at 534 nm Ohkawa *et al.* (1979).

Serum cholesterol and triglycerides levels

Serum cholesterol and triglycerides levels was determined using kits of Biodiagnostic®, Cairo, Egypt. As described by Richmond (1973) and Fossati and Prencipe (1982), respectively.

Liver enzymes and kidney functions

Serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were carried out according to Reitman and Frankel (1957).

Serum creatinine and urea levels were determined by colorimetric method according to the technique developed by Husdan and Rapoport (1968) and Fawcett and Scott (1960), respectively.

Statistical analysis

The tabulated data are expressed as mean ± standard deviation (mean ± SE). The data were statistically analyzed by using analysis of variance (ANOVA), the difference was considered significant when ($P < 0.05$) using SPSS 27 (IBM. NY, USA).

Results

Characterization of ZnO-NPs

X-ray diffractometer (XRD)

Figure 1 illustrates the XRD curve of the synthesized ZnO NPs. Results can be observed from XRD pattern illustrated the high diffraction peaks intensity, which indicated the high crystalline structure of the prepared ZnO NPs. The characteristic diffraction peaks of ZnO NPs are dominated diffraction peaks in the XRD curve according to Brucker database COD no. 2300113 with hexagonal crystal. However, the diffraction peaks corresponding to the (100), (002), (101), (102), (110), (103), (200), (112), (201), (004) and (202) planes. In addition to, the oxygen element represents 20.8% while the Zinc element represents 79.2% of the sample.

Scanning and transmission electron microscope (SEM & TEM)

The SEM and TEM results confirmed that ZnO NPs Encased in nanospheres of uniform spherical shape and size (Figure 2 and 3).

Dynamic light scattering (DLS)

The synthesized ZnO NPs was examined for its size distribution by dynamic light scattering (DLS) method and have been exposed to possible zeta estimates. Figure 4 illustrated the homogenous size distribution and the mean size of ZnO NPs was ~33 nm, which compatible with the

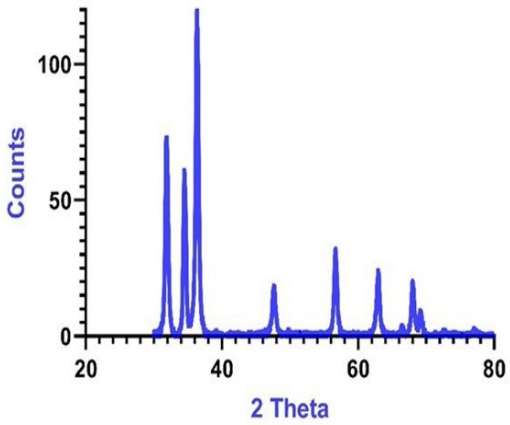

Fig. 1. Illustrates the fingerprint of XRD pattern of ZnO NPs according to Brucker database COD no. 2300113.

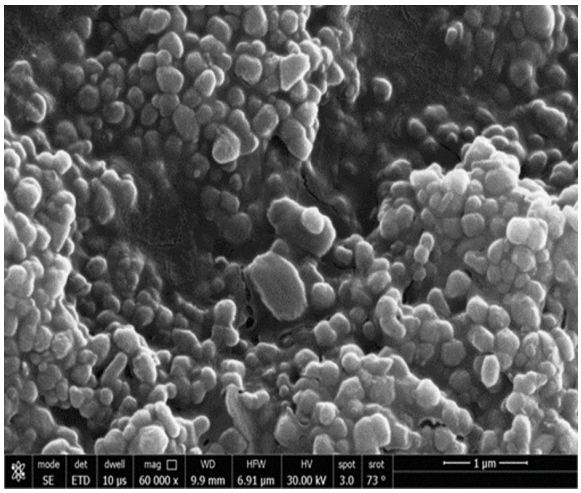

Fig. 2. Illustrate the spherical monodispersed ZnO-NPs SEM image.

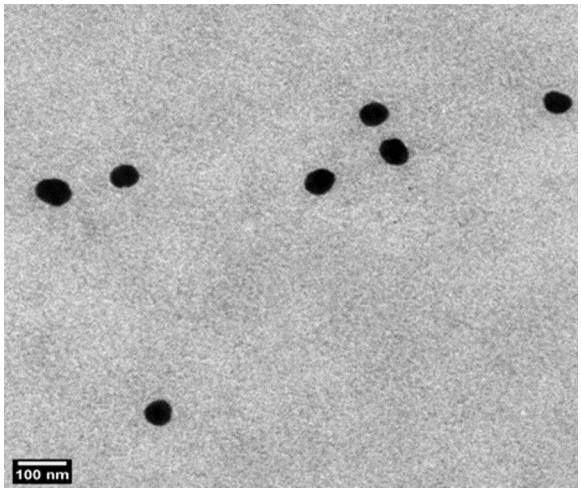

Fig. 3. Illustrate the TEM image of ZnO-NPs nanospheres.

Table 1. Cumulative mortality rate among different experimental groups.

	G1	G2	G3	G4	G5	G6	G7	G8	G9
1st week	0	0	0	0	0	0	0	0	0
2nd week	1	0	0	0	1	0	0	0	0
3rd week	2	0	1	0	1	0	1	0	0
4th week	2	0	1	0	1	0	0	0	0
5th week	0	0	0	0	0	0	0	0	0
Cumulative mortalities	5/21(23%)	0/21	2/21(9.5%)	0/21	3/21(14%)	0/21	1/21(4.7%)	1/21(4.7%)	0/21

mean size observed in the TEM and SEM pictures investigation.

Zeta's expected advantages of ZnO NPs were found to be – 34.01 mV (Figure 5). It can therefore be concluded from the results that the high value of the zeta potential lead to higher colloidal stability in water and hence can be derived from the high bioactivity of the ZnO NPs towards different bacterial species.

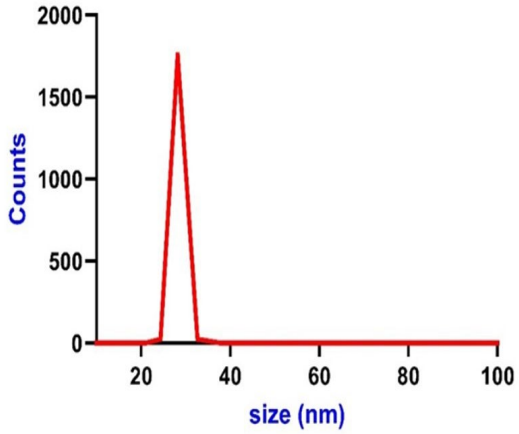

Fig. 4. The DLS pattern of ZnO NPs.

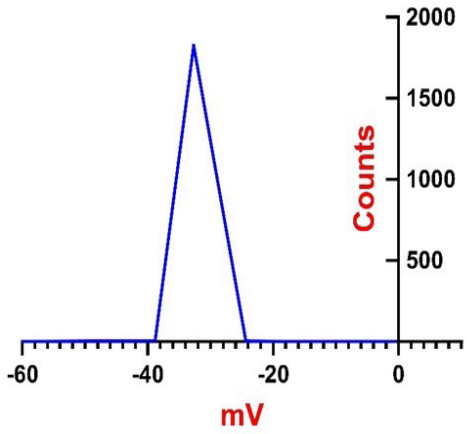

Fig. 5. Zeta potential pattern of ZnO NPs.

In vitro antimicrobial evaluation

The results revealed that the MIC for ZnO-NPs was 0.0312 µg/ml and 0.062 µg/ml against MS and MG, respectively while, MIC for Difloxacin was 0.125 µg/ml and 0.125 µg/ml against MS and MG, respectively.

In vivo antimicrobial evaluation

Cumulative mortalities

As observed in Table 1, the highest cumulative mortality rates have been observed in control positive groups G1 23% (5/21), G5 14% (3/21) followed by G3 9.5% (2/21) then G7 and G8 showed the same mortality rate 4.7% (1/21) while, G2, G4, G6 and G9 showed no mortalities all over the experiment. It has been observed that the groups treated with 1% ZnO-NPs revealed no mortalities and that concentration of Zo-NPs succeeded in stopping the mortalities of the challenged birds.

Clinical signs score

Figure 6 showed that G1 showed a higher significant increase in the severity of clinical signs among MS challenged groups followed by G4 while birds treated with 1% ZnO-NPs in G1 revealed a significant reduction in the severity of clinical signs in contrast with G1. On the other hand, in groups challenged with MG, G5 showed a higher significant increase in the severity of clinical signs among MG challenged groups followed by G8 while birds treated with 1% ZnO-NPs in G6 revealed a significant reduction in the severity of clinical signs in contrast with G5. All the experimental groups showed a significant increase in the severity of clinical signs when they were contrasted with G9 at p value ≤0.05.

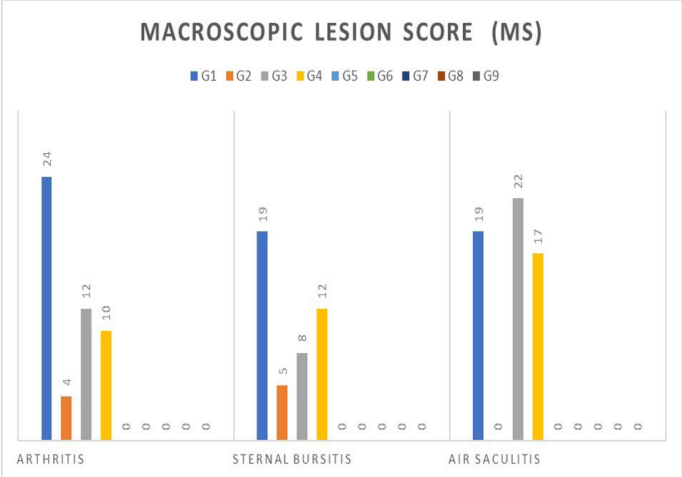

Fig. 6. Macroscopic lesion score among different MS challenged experimental groups.

Macroscopic lesion score

As shown in Figure 6 in groups challenged with MS, the severity of arthritis showed a significant increase in its lesion score in G1 followed by G3 and G4. Birds treated with 1% ZnO-NPs revealed a significant reduction in the lesion score in contrast with G1. Sternal bursitis lesion score revealed a significant elevation in G1 followed by G4 then G3 and the smallest lesion score was recorded in G2. Air saculitis lesion score revealed a significant increase in G3 followed by G1 then G4 while no air saculitis lesion score has been recorded in G2.

As presented in Figure 7 in groups challenged with MG, the severity of sternal bursitis showed a significant increase in its lesion score in G5 followed by G8 and G7. Birds treated with 1% ZnO-NPs in G6 revealed a significant reduction in the lesion score in contrast with G5. The air saculitis lesion score revealed a significant elevation in G5 followed by G8 then G7 and the smallest lesion score was recorded in G6. Lung lesion score showed a significant elevation in G5 followed by G7 and G8 (that have the same lung lesion score) then G6 that showed the smallest lung macroscopic lesion score when contrasted with G5. All challenged birds

Table 2. The impact of different treatments on birds' performance.

Parameters	Control G1	Experimental groups								*p*-value
		G2	G3	G4	G5	G6	G7	G8	G9	
IBW (g)	44.5±0.2^{a}	42.6±0.2^{a}	44.6±0.2^{b}	45.2±0.1^{b}	45.10±0.3^{b}	45.0±0.02^{b}	45.0±0.01^{b}	45.0±0.01^{b}	45.0±0.01^{b}	≤0.05
FBW (g)	1306.8±1.9^{a}	1727.6±0.9^{b}	1902±0.7^{c}	1502±0.8^{d}	1392.8±1.2^{a}	1952±0.7^{c}	1905.6±1.7^{c}	1552.2±0.8^{d}	2102.6±0.9^{e}	≤0.05
FI (g)	3102±0.7^{a}	3333±0.7^{d}	3102.8±0.8^{a}	3102.4±1.02^{a}	3202±0.7^{b}	3201±0.5^{b}	3252±0.7^{c}	3102.6±0.9^{a}	3502±0.7^{e}	≤0.05
FCR (g/ g)	2.46±0.01^{a}	1.8±0.01^{f}	1.87±0.01^{e}	2.13±0.01^{c}	2.37±0.01^{b}	1.8±0.01^{f}	1.8±0.007^{f}	2.1±0.007^{d}	1.62±0.04^{g}	≤0.05

Initial body weight (IBW), Final body weight (FBW), Feed intake (FI), Feed conversion ratio (FCR), Data are presented mean ±SD, p-value ≤0.05 indicate significant difference.

either with MS or MG revealed a significant increase in the different macroscopic lesions score when contrasted with G9.

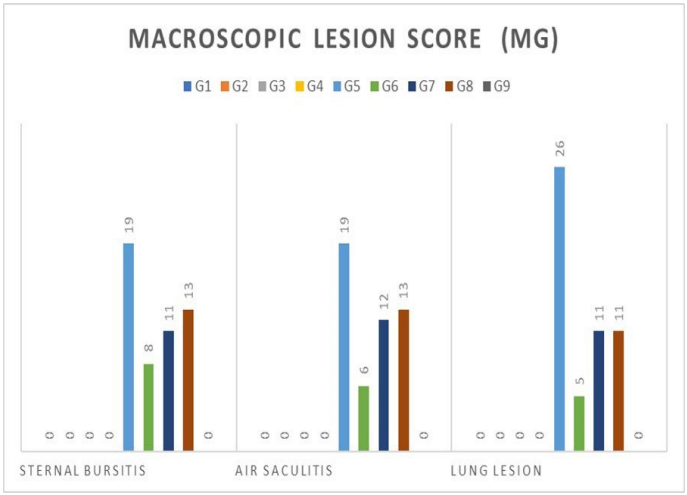

Fig. 7. Macroscopic lesion score among different MS challenged experimental groups.

Evaluation of bird performance

Body weight (BW) and feed conversion rate (FCR)

As demonstrated in Table 2, FBW revealed significant decrease in G1(1306.8±1.9) and G2 (1392.8±1.2) at p-value ≤0.05 in comparison with G9 (2102.6±0.9) followed by G4 (1502±0.8) then G8 (1552.2±0.8). G3 (1902±0.7), G6 (1952±0.7) and G7 (1905.6±1.7) showed better FBW and there was no significant difference between these three groups while these groups showed a significant increase in FBW in comparison with G1 (1306.8±1.9) and G5 (1392.8±1.2) at p-value ≤0.05.

Regarding the FCR, there was a significant increase in FCR in G1 (2.46±0.01) followed by G5 (2.37±0.01) then G4 (2.13±0.01) and G8 (2.1±0.007) in comparison with G9 (1.62±0.04) at p-value ≤0.05. There was no significant difference between FCR recorded among G2 (1.8±0.01), G6 (1.8±0.01) and G7 (1.8±0.007) but these three groups showed better FCR when compared with G1 (2.46±0.01) and G5 (2.37±0.01). G3 revealed a significant improvement in FCR (1.87±0.01) when compared with G1(2.46±0.01) and G5 (2.37±0.01) at p-value ≤0.05.

Serum antioxidant status

Table 3 presented the impact of different treatments on the birds' antioxidant status. Serum catalase (U/ml) activity showed a significant decrease in G1 (21.3±0.1) and G5 (20.5±0.07) when compared with G9 (30.34±0.07) at p-value ≤0.05. there was no significant difference between G3 (32.2±0.1), G5 (20.5±0.07) and G6 (35.32±0.05) at p-value ≤0.05. Also, no significant difference has been recorded between G4 (30.3±0.1), G7 (30.14±0.05) and G8 (30.34±0.06) but these three groups showed a significant in serum catalase level when compared with G1 (21.3±0.1) and G5 (20.5±0.07) at p-value ≤0.05.

The GSH levels revealed a significant decrease in G1(24.2±0.1) and G5 (28.5±0.001) when compared with G9 (43.2±0.08) at p-value ≤0.05. there was no significant difference between G3 (32.5±0.001) and G8 (29.3±0.08) at p-value ≤0.05. While there was a significant increase in serum GSH levels in G2 (32.6±0.05) and G4 (39.2±0.001) when compared with G1 (24.2±0.1). Also, G6 (31.1±0.001) and G7 (39.2±0.08) showed a significant elevation in serum GSH levels when compared with G5 (28.5±0.001) at p-value ≤0.05.

The MDA serum level showed no significant difference between G1 (27.62±0.05a), G2 (19.4±0.001), G4 (16.7±0.05), G5 (27.62±0.1), G7 (17.62±0.1) and G8 (19.24±0.2) but these groups revealed a significant difference when compared with control negative birds in G9 (14.62±0.2) at p-value ≤0.05. Also, G3 (19.1±0.001) and G6 (18.3±0.06) showed a significant decrease in MDA levels when contrasted with G1 (27.62±0.05) and G5 (27.62±0.1) at p-value ≤0.05.

Blood lipid profile

As presented in Table 4, the serum cholesterol levels showed a significant decrease G2 (77.5±0.6), G3 (132.2±0.5) and G4 (127.6±0.8) when compared with G1 (174.3±0.7) at p-value ≤0.05. Also, G6 (104±0.6), G7 (132±0.7) and G8 (127±0.66) revealed a significant decrease in serum cholesterol levels when compared with G5 (192±0.65) at p-value ≤0.05. All treated groups from G1 to G8 showed a significant higher serum cholesterol level when contrasted with G9 (63.8±0.70.58) at p-value ≤0.05. The serum triglycerides levels showed a significant increase in G1 (146.26±0.07), G4 (146.28±0.08), G5 (165.28±0.08), G6 (120.2±0.07), G7 (124.3±0.07) and G8 (145.2±0.67) when compared with G9 (70.3±0.07) at p-value ≤0.05. The best serum triglycines levels was recorded in G2 (89.2±0.07) treated with 1% Zo-NPs and this group showed no significant difference when contrasted with control negative birds in G9 (70.3±0.07) at p-value ≤0.05.

Liver enzymes and kidney function

As shown in Table 5, liver enzymes, serum ALT levels showed a significant increase in G1 (57.4±0.1) and G5 (35.24±0.09) when compared with G9 (11.3±0.1) at p-value ≤0.05. G2 (20.3±0.07), G3 (17.5±0.06) and G4 (40.2±0.07) revealed a significant reduction in serum ALT levels when contrasted with G1 (57.4±0.1) at p-value ≤0.05. Also, G6 (17.24±0.08),

Table 3. The impact of different treatments on antioxidant status.

Antioxidant parameters	Experimental groups									*p*-value
	G1	G2	G3	G4	G5	G6	G7	G8	G9	
Catalase (U/ml)	21.30±0.1^{b}	29.3±0.07^{d}	32.2±0.1^{a}	30.3±0.1^{e}	20.5±0.07^{a}	35.32±0.05^{a}	30.14±0.05^{e}	30.34±0.06^{e}	30.34±0.07^{c}	
MDA (ng/ml)	27.62±0.05^{a}	19.4±0.001^{a}	19.1±0.001^{f}	16.7±0.05^{a}	27.62±0.1^{a}	18.3±0.06^{g}	17.62±0.1^{a}	19.24±0.2^{a}	14.62±0.2^{b}	≤0.05
GSH (mg/dl)	24.20±0.1^{g}	32.6±0.05^{f}	32.5±0.001^{e}	39.2±0.001^{b}	28.5±0.001^{g}	31.1±0.001^{d}	39.2±0.08^{c}	29.3±0.08^{e}	43.2±0.08^{a}	

Malondialdehyde (MDA), Glutathione (GSH).

Table 4. The impact of different treatments on blood lipid profile.

Parameters	Experimental groups									*p*-value
	G1	G2	G3	G4	G5	G6	G7	G8	G9	
Cholesterol (mg/dl)	174.3±0.7^{g}	77.5±0.6^{b}	132.2±0.5^{d}	127.6±0.8^{c}	192±0.65^{H}	104±0.6^{a}	132±0.7^{d}	127±0.66^{c}	63.8±0.70.58^{a}	≤0.05
Triglycerides (mg/dl)	146.3±0.07^{ab}	89.2±0.07^{a}	120.5±0.56^{b}	146.3±0.08^{ab}	165.3±0.08^{ab}	120.2±0.07^{ab}	124.3±0.07^{ab}	145.2±0.67^{ab}	70.3±0.07^{a}	

Table 5. The impact of different treatments on liver and kidney functions.

Parameters	Experimental groups									*p*-value
	G1	G2	G3	G4	G5	G6	G7	G8	G9	
Creatinine (mg/dl)	0.82±0.001^{g}	0.25±0.001^{b}	0.27±0.001^{c}	0.33±0.001^{d}	0.44±0.001^{f}	0.25±0.001^{b}	0.36±0.001^{e}	0.33±0.001^{d}	0.16±0.001^{a}	
Urea (mg/dl)	17.2±0.07^{g}	10.2±0.07^{b}	12.3±0.07^{d}	14.2±0.07^{f}	13.14±0.074^{e}	12.12±0.05^{d}	11.2±0.07^{c}	14.22±0.086^{f}	9.2±0.07^{a}	≤0.05
ALT (U/L)	57.4±0.1^{g}	20.3±0.07c	17.5±0.06^{b}	40.2±0.07^{f}	35.24±0.09^{e}	17.24±0.08^{b}	23.22±0.05^{d}	40.2±0.07^{f}	11.3±0.1^{a}	
AST (U/L)	356±0.7^{g}	203±0.7^{d}	172±0.6^{bc}	222±0.7^{e}	282±0.6^{f}	173±0.5^{c}	170±0.08^{b}	223±0.7^{e}	102±0.9^{a}	

Alanine transaminase (ALT), Aspartate aminotransferase (AST).

and G7 (23.22±0.05) showed a significant reduction in serum ALT levels when compared with G5 (35.24±0.09) at p-value ≤0.05. All treated groups showed a significant increase in serum ALT levels when contrasted with control negative birds in G9 (11.3±0.1) at p-value ≤0.05.

Serum AST levels showed a significant increase in G1 (356±0.7) and G5 (282±0.6) when contrasted with G9 (102±0.9) at p-value ≤0.05. G2 (203±0.7), G3 (172±0.6) and G4 (222±0.7) revealed a significant reduction in serum AST levels when contrasted with G1 (356±0.7) at p-value ≤0.05. Also, G6 (173±0.5), G7 (170±0.08) and G8 (223±0.7) showed a significant reduction in serum AST levels when contrasted with G5 (282±0.6) at p-value ≤0.05. All treated groups revealed a significant elevation in serum AST levels when contrasted with control negative birds in G9 (102±0.9) at p-value ≤0.05.

Regarding kidney function tests in the form of creatinine level showed a significant increase in G1 (0.82±0.001) and G5 (0.44±0.001) when compared with G9 (0.16±0.001) at p-value ≤0.05. G2 (0.25±0.001), G3 (0.27±0.001) and G4 (0.33±0.001) revealed a significant reduction in serum creatinine levels when contrasted with G1 (0.82±0.001) at p-value ≤0.05. Also, G6 (0.25±0.001), G7 (0.36±0.001) and G8 (0.33±0.001) showed a significant reduction in serum creatinine levels when compared with G5 (0.44±0.001) at p-value ≤0.05. All treated groups showed a significant increase in serum creatinine levels when contrasted with control negative birds in G9 (0.16±0.001) at p-value ≤0.05. Urea levels showed a significant increase in G1 (17.2±0.07) and G5 (13.14±0.074) when compared with G9 (9.2±0.07) at p-value ≤0.05. G2 (10.2±0.07), G3 (12.3±0.07d) and G4 (14.2±0.07) revealed a significant reduction in urea levels when contrasted with G1 (17.2±0.07) at p-value ≤0.05. Also, G6 (12.12±0.05), G7 (11.2±0.07) and G8 (14.22±0.086) showed a significant reduction in serum creatinine levels when compared with G5 (13.14±0.074) at p-value ≤0.05. All treated groups showed a significant increase in serum creatinine levels when contrasted with control negative birds in G9 (9.2±0.07) at p-value ≤0.05.

Discussion

Avian *Mycoplasma* is the most harmful, inflicting enormous losses for the poultry sector (Marouf *et al*., 2022b). However, the application of biosecurity measures side by side with vaccination programs, avian *Mycoplasma* continues to adapt to the poultry production sector and can evade the bird immunity that allow its persistent especially high populated poultry zones (Bottinelli *et al*., 2022). Also, due to the enormous avian populations, multi-age farms, and numerous potential links (people, feed trucks, etc.) among the meat and layer sectors, complete control of avian *Mycoplasma* is difficult to achieve (Kleven, 2008). Antimicrobial treatment may be beneficial in some situations when managing an outbreak but because long-term usage of antimicrobial medications can result in the formation of anti-Mycoplasma drug resistance (Taiyari *et al*., 2021). Macrolides, tetracyclines, pleuromutilins, and fluoroquinolones are the antimicrobials that are thought to be most useful in treating Mycoplasmas and are therefore the most frequently utilized however, *Mycoplasma* infections may respond to treatment slowly because only fluoroquinolones have a bactericidal impact, while the others typically just have a bacteriostatic effect one (Hofacre *et al*., 2013).

In this study, ZnO-NPs showed a potent *in vitro* and *in vivo* anti-Mycoplasma activity. The *in vivo* efficacy of ZnO-NPs especially with a concentration of 1% appeared in the shape of reduction of cumulative mortalities in either groups challenged with MS or MG, improvement the FCR in the treated birds and groups treated with 1% ZnO-NPs revealed a significant reduction in clinical sins and macroscopic lesion score. These findings are parallel to that reported by Fathi *et al*. (2019) who confirmed that ZnO-NPs is a potent therapy against *Mycoplasma bovis* induced mastitis in experimental rabbits. In addition to different studies that confirmed the wide range of antibacterial activity of ZnO-NPs against most gram-positive and gram-negative bacteria (Wang *et al*., 2012; Kadiyala *et al*., 2018). Xie *et al*. (2011) owed the antibacterial activity (bactericidal effect) of ZnO-NPs to its ability to disrupt the bacterial cell membrane, induction of intercellular reactive oxygen species that considered as strong oxidizing elements against pathogenic bacteria, rise bacterial cell membrane permeability and destruct bacterial membrane integrity.

Aside from its participation in growth, metabolic pathways, and physiological and biosynthetic activities within the bodies of animals and poultry, zinc also performs other essential functions (Cesur *et al*., 2005). From our data, birds treated with ZnO-NPs in G2 and G6 revealed a significant increase in FBW, and they revealed a significant improvement in FCR. From our result, ZnO-NPs treated birds showed better performance (FBW, FCR) in comparison with antibiotic treated birds in G4, G8 as well as control negative birds. These results are in concur with Ahmadi *et al*. (2013) who noticed that the addition of ZnO-NPs in a level 30 to 90 mg/kg of diet of broiler chickens improved their growth performance. These results may be contributed to the fact that zinc is involved in several enzymatic and metabolic processes in animals' bodies and is necessary for the action of around 250 to 300 enzymes (Prasad and Kucuk, 2002).

From our results birds supplied with Zn-NPs IN G2, G3, G6 and G7 showed a significant improvement in antioxidant status (catalase, GSH & MDA) levels in contrast with G1 and G5. Also, it was previously confirmed that the dietary ZnO-NPs decreases the MDA level and elevates superoxide dismutase (SOD) and catalase (CAT) activities in birds (Zhao *et al*., 2014; El-Bahr *et al*., 2020). Zinc is one of the building blocks of different proteins and dependent enzymes like superoxide dismutase (SOD) that act as a key element of antioxidant defense mechanism (Bao *et al*., 2009). Also, according to research, Zn promotes the production of several types of metallothionein, a cystine-rich protein that functions as a free radical scavenger (Oteiza *et al*., 1996).

From our observations birds in G2 and G6 revealed a significant improvement in lipid profile (cholesterol and triglycerides) levels when contrasted with other experimental groups. In this context, the dietary inclusion of ZnO-NPs in poultry diet resulted in a lowering in serum total cholesterol (TC) and triacylglycerol (TAG) as it improves fat metabolism or lowering the absorption of dietary fats (Ahmadi *et al*., 2013).

In this study, addition of different levels of ZnO-NPs resulted in a significant improvement in liver (AST and ALT) levels and, it was noticed that addition of 1% ZnO-NPs resulted in a significant improvement in liver function. That could be contributed to that zinc participates in the activity of several liver enzymes as a cofactor, such as alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), and aspartate aminotransferase (AST) (Bennett *et al*., 2001) and different studies confirmed that lowering the zinc level in the blood plasma is correlated to the impaired physiological duties and different of hepatic disorders as hepatitis and liver cirrhosis (Cesur *et al*., 2005).

Creatinine, urea, and blood urea nitrogen are indicators of renal function (Hussain *et al*., 2018). In this study, the addition of different levels of ZnO-NPs resulted in a significant improvement in renal functions (creatinine and urea) levels. From our results, 1% ZnO-NPs revealed a significant improvement in the kidney functions in the treated birds. Hussain *et al*. (2018) found similar results and confirmed that ZnO-NPs has a hepato-renal protective impact in the treated mice. Also, Abdel-Wareth *et al*. (2022) noticed that dietary addition of zinc oxide ZnO-NPs at levels of 20, 40, and 60 mg/kg ensued in better bird productivity, digestion of nutrient, carcass traits, and hepatic and renal functions of broiler chickens exposed to thermal stress.

Conclusion

MIC test should be adopted as a routine to choose the most suitable anti-Mycoplasma drug to avoid the existence of anti-Mycoplasma drug

resistance. ZnO-NPs exhibited a significant *in vitro* and *in vivo* antibacterial impact against avian mycoplasmosis with optimum responses at the level of 1%. The usage of ZnO-NPs improves birds growth performance, enhances the liver and kidney functions, lowers serum cholesterol & triglyceride levels, boosted the antioxidant status of the treated broiler chickens.

Conflict of interest

The authors declare that they have no conflict of interest.

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