Use of biological nano zinc as a feed additive in quail nutrition: biosynthesis, antimicrobial activity and its effect on growth, feed utilisation, blood metabolites and intestinal microbiota

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ABSTRACT
A total of 200 1-week-old Japanese quails were randomly allocated into five treatment groups, each consisting of 40 unsexed birds (five replicates, eight birds each). Quails were reared in traditional cages (90 x 40 x 40 cm), and feed and water were made available throughout the 5-week trial. The treatment groups were as follows: the 1st was fed basal diet, whereas the 2nd, 3rd, 4th, and 5th groups were fed with ration supplemented with nano zinc (Zn-NPs) at doses of 0.1, 0.2, 0.3, and 0.4 g/kg diet, respectively. Results showed that, a significant (p < 0.0001) improvement in body weight, weight gain, feed intake, and feed conversion ratio was observed in birds fed diets supplemented with 0.2 g/kg of Zn-NPs. Supplementation of Zn-NPs at doses of 0.1 – 0.3 g/kg diet demonstrated a positive impact on the activity of ALT, AST, and LDH. The liver profile parameters were not statistically influenced (p > .05) by dietary biological nano zinc, with the exception of total cholesterol (TC), high density lipoprotein (HDL), and low density lipoprotein (LDL). Also, dietary supplementation of biological Zn-NPs at concentrations of 0.1 – 0.3 g/kg diet demonstrated a positive impact on superoxide dismutase (SOD), glutathione peroxidase (GPX), malondialdehyde (MDA), immunoglobulin G (IgG), and immunoglobulin M (IgM). Dietary supplementation of Zn-NPs led to an increase in beneficial microbial populations. From the obtained results, Zn-NPs supplementation at 0.2 g/kg diet had a positive effect on the performance and physiological status of growing Japanese quails.

HIGHLIGHTS

- Zinc (Zn) is an important trace element for the general functioning of the animal body.
- A significant improvement in growth performance was observed in birds fed diet enriched with 0.2 g/kg of Zn-NPs.
- Supplementation of Zn-NPs (0.1 – 0.3 g/kg diet) demonstrated a positive impact on immunity and antioxidant indices.

Introduction
Feed supplements and additives have been effectively used in poultry rations for increasing the productivity and improving the general health and wellbeing (Yatoo et al. 2017; Mohamed et al. 2019; Alagawany et al. 2020a, 2020b). Many recent technologies are being widely used in improving poultry production in terms of quantity and quality, such as the use of nanoparticles (Sahoo et al. 2016; El-Rayes et al. 2019; Nabi et al. 2020; Reda et al. 2020). Among the metal nanoparticles (NP) annually produced in the world, zinc oxide nanoparticles (ZnO-NPs) are the third largest in terms of size, and rank after Nano-SiO2 and Nano-TiO2 in terms of their multi-functional physical and chemical properties and their easy synthesis (Wahab et al. 2013; Attia et al. 2019). With the emergence of nanotechnology, zinc can be added as a feed supplement in many forms to improve the efficiency of trace minerals in poultry and livestock (Attia et al. 2013; Geetha et al. 2020).

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Zinc (Zn) is an important trace element for the general functioning of the animal body. It has three essential biological functions, namely, catalytic roles in the functioning of more than 300 enzymes, structural roles, and regulatory roles. Moreover, it influences the immune system, nucleic acid synthesis, cell proliferation, protein synthesis, protein and carbohydrate metabolism, and enzymatic activities in the living system (Thati et al. 2010).

Studies have already proven the dose-dependent effect of ZnO-NPs on the growth performance and physiological status of livestock and poultry (Sahoo et al. 2016; Mahmoud et al. 2020). In addition, ZnO has been listed as a “Generally Recognized as Safe” compound by the US Food and Drug Administration due to its non-toxic properties (FDA 21CFR182.8991). It is hypothesised that the use of biological nano zinc in the diets is expected to have beneficial effects on performance, feed utilisation, and health aspects of Japanese quail. Therefore, this study aimed to evaluate the antibacterial and antifungal effect of biological nano zinc and its role in improving the performance and physiological status of growing Japanese quails.

Materials and methods

This experiment was designed at Poultry Department and carried out at Poultry Research Farm, Faculty of Agriculture, Zagazig University, Egypt.

Biosynthesis of zinc nanoparticles that were used in this study

Zinc nanoparticles (ZnO-NPs) were biosynthesized by Bacillus subtilis AM12 that was isolated from a soil sample obtained from Zagazig City, Sharkia Governorate, Egypt (El-Saadony et al. 2019; 2020). The optimal conditions for the manufacture of zinc nanoparticles were as follows: zinc nitrate concentration of 200 mg/L, nutrient broth medium, pH 7, incubated in a shaking incubator with an agitation speed of 130 xg for 72 h, and the temperature was 30 °C. To biofabricate ZnO-NPs, 10 mL of the Bacillus subtilis AM12 supernatant was mixed with 90 mL of zinc nitrate (Zn(NO₃)₂) solution (200 mg/L) (Mishra et al. 2013). The reaction mixture was then incubated at shaking incubator. The control used was (Zn(NO₃)₂) dissolved in sterilised deionised water and incubated under the same conditions. Experiments were carried out in triplicate. A visible change was observed in the colour of the (Zn(NO₃)₂) + B. subtilis AM12 supernatant, changing from colourless to white after 72 h; this was taken as an initial indicator to biosynthesis of the ZnO-NPs. No colour change was observed in the control, indicating that the bacterial supernatant was responsible for the biosynthesis of zinc nanoparticles. Zinc nanoparticles that were obtained from the (Zn(NO₃)₂) + B. subtilis AM12 supernatant was then characterised using standard analytical techniques. The ultraviolet-visible (UV-Vis) spectrum showed an absorption peak at 320 nm. Transmission electron microscopy showed that the mean diameter of the formed ZnO-NPs was 22–43 nm. Powder X-ray diffraction showed that the ZnO-NPs are crystalline in nature, and that they have a spherical structure. The average ZnO-NP size was 25.31 nm, and the zeta potential was determined to be −28.7 mV. Fourier Transform Infra-red Spectroscopy analysis (FT-IR) confirmed the presence of active groups such as alcohols, phenols, alkenes, and amines that are responsible for the bioreduction capabilities and stabilisation of ZnO-NPs in the reaction mixture.

Antibacterial activity of ZnO-NPs

The ZnO-NPs were homogenised in deionised water to test their antibacterial activity through a disc assay. Six pathogenic bacteria, namely, Gram-positive Bacillus subtilis ATCC 6633, Listeria monocytogenes ATCC 15313, and Staphylococcus aureus ATCC 8538, as well as Gram-negative Salmonella enterica ATCC 35664, Escherichia coli ATCC 8739, and Klebsiella pneumonia ATCC 27736, were used for this assay. Petri plates were filled with 20 ml of melted Muller Hinton agar (MHA) medium and were allowed to solidify. The tested strains were activated on nutrient broth for 24 h prior to the experiment. Afterwards, bacterial inoculum (100 μL) was spread onto the MHA plates; this was done in triplicate. Paper discs (5 mm in diameter) saturated with different concentrations of ZnO-NPs and only one concentration of ciprofloxacin (1 g) were (20, 40, 80, 160, and 320 μg/mL) placed on hardened MHA petri plates. The plates were incubated for a day at 37 °C, and the inhibition zone diameters were measured. MIC was evaluated in MH broth using a micro dilution method. Ciprofloxacin was used as the reference antibiotic standard for antibacterial activity, and zinc nitrate was used as positive control. MIC was determined as the least ZnONPs concentration that inhibited bacterial growth after a day of incubation. The tested bacterial strains were sub-cultured on MH broth and were incubated under sterile conditions at 37 °C for 24 h. Different concentrations of ZnO-NPs (20, 40, 80, 160, and 320 μg/mL) were homogenised in 10 mL MH broth in test tubes. The test tubes
containing NH broth without ZnO-NPs and sterilised deionised water were used as negative and positive controls, respectively. All tubes were inoculated with each bacterial inoculum and were then incubated for 24 h at 37°C, then the turbidity was measured in triplicate at 600 nm using a spectrophotometer. The MBC, on the other hand, was identified by sub-culturing MIC tubes on MHA plates then incubated at the same conditions and the lowest concentration of ZnO-NPs didn’t appear any growth was defined as the MBC (Holla et al. 2012).

**Antifungal activity of ZnO-NPs**

Biologically synthesised ZnO-NPs were tested against the fungal isolates *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum*, *Alternaria alternate*, and *Penicillium expansum* to determine antifungal activity. In potato dextrose agar (PDA) fungal cells were grown for 5 days at 28°C, and standard size of spore suspension of $1 \times 10^6$ colony-forming units/mL were grown on fresh solid PDA medium. The agar well diffusion method (NCCLS, 2002) was then carried out. Wells with a diameter of 6 mm were punched into the PDA medium and were filled with 50 μl of various ZnO-NP concentrations (20, 40, 80, 160, and 320 μg/mL) and were incubated for 5 days at 28°C. Under the same conditions, controls were wells filled with zinc nitrate. The MIC values of the ZnO-NPs were calculated as the least concentration that decreased fungal growth. The antifungal activity was measured against the final $10^6$ colony-forming units (CFU)/mL fungal concentration. All tests were conducted three times.

**Animals, design and diets**

A total of 200 Japanese quails that were a week old were randomly allocated into 5 treatment groups. Each group consists of 40 unsexed birds; five replicate groups were made comprising eight birds each. Quails were reared in traditional cages (90 × 40 × 40 cm), and mash feed and water were freely available throughout the five-week trial. The treatment groups were as follows: the 1st was fed a basal diet, while the 2nd, 3rd, 4th, and 5th groups were fed with ration supplemented with nano zinc at doses of 0.1, 0.2, 0.3, and 0.4 g/kg diet, respectively. The basal ration was formulated to meet quail requirements which contained 24% protein, 12.53 MJ/kg, 0.80% calcium, 0.45% phosphorus, 1.30% lysine and 0.92% total sulphur amino acids (TSAA). Birds were reared in conventional type cage (50 × 30 × 50 cm³; 1500 cm² of floor space) with water and feed provided *ad libitum*. Birds were subjected to 17 h light: 7 h dark cycle throughout the experimental period.

**Growth performance and carcase measurements**

All parameters related to growth (feed intake, conversion, body weight, and weight gain) were evaluated when the quails reached 1, 3, and 5 weeks old. For carcase parameters, at the end of the trial, 25 quails were randomly taken (5 per treatment), weighed, and slaughtered after fasting for 6 h. All the edible parts (liver, heart, gizzard, and eviscerated carcass) were weighed, and the results were expressed as % of pre slaughter weight.

**Blood chemistry**

At the end of the trial, the 5-week-old quails were slaughtered and we collected their blood into heparinised tubes. Hematological parameters (LYM: lymphocytes; WBCs: white blood cells; MID: mid-range; RBCs: red blood cells; GRA: granulocytes; HGB: haemoglobin; MCV: mean corpuscular volume; HCT: haematocrit; PLT: platelet count; MCH: mean corpuscular haemoglobin) were measured. In terms of biochemical parameters (TP: total protein; Alb: albumin; AST: aspartate aminotransferase and ALT: alanine aminotransferase. TC: total cholesterol; LDH: lactate dehydrogenase; TG: triglycerides; LDL: low density lipoprotein; VLDL: very-low-density lipoprotein; HDL: high density lipoprotein; SOD: superoxide dismutase; MDA: malondialdehyde; GPX: glutathione peroxidase; IgG and M: immunoglobulin G and M), the samples were centrifuged (Janetzki, T32c, 5000 rpm, Germany) at 2146.56 g for 15 min to separate the plasma. We determined all the biochemical blood indices using commercial kits from Biodiagnostic Company (Giza, Egypt).

**Microbiological analysis**

At the end of the trial, samples (~10 g) were obtained from the caecum (five samples per treatment) and were transferred to a 250 mL Erlenmeyer flask containing 90 mL of peptone (0.1% peptone) in saline solution (0.85%NaCl) and were thoroughly mixed. The total bacterial count (TBC), *Enterococcus* spp. count, total count of yeasts and molds (TYMC), lactic acid bacteria count, coliform, *E. coli* count, and *Salmonella* spp. count, were estimated as per (Reda et al. 2020). The number of bacterial and fungal counts was converted into log numbers.
**Statistics**

All of the statistical analyses were performed using SAS. The growth, carcase yield, hematological parameters, lipid profile, liver and kidney functions, immunological indices, antioxidants status, and microbiology data were analysed using one-way ANOVA. Orthogonal polynomial contrasts (linear and quadratic) were used to test the significance of the different doses of dietary Bio-Zn-NPs.

**Results and discussion**

**Antibacterial activity of ZnO-NPs**

The antibacterial impact of ZnO-NPs bio-fabricated by the supernatant of *Bacillus subtilis* AM12 was examined using a disc assay against six pathogenic bacterial strains, namely, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 15313, *Staphylococcus aureus* ATCC 8538, *Salmonella enterica* ATCC 35664, *Escherichia coli* ATCC 8739, and *Klebsiella pneumoniae* ATCC 27736. The obtained IDZs (mm) are shown in Table 1. Gram-positive bacteria were more susceptible to biosynthesized ZnO-NPs than Gram-negative bacteria. The diameters were arranged according to their sensitivity was observed in those with ciprofloxacin (24.7 ± 0.5); moreover, a slight increase (8%) in bacteriostatic sensitivity was observed in those with ciprofloxacin (20.4 ± 0.5); furthermore, a slight increase (8%) in bacteriostatic sensitivity was observed in those with ciprofloxacin (20.4 ± 0.5); however, a slight increase (8%) in bacteriostatic sensitivity was observed in those with ciprofloxacin (20.4 ± 0.5); therefore, ZnO-NPs may be a treatment choice for bacterial infections in the hospital. The obtained MIC and MBC results of each bacterial isolate were different to those obtained by Gunalan et al. (2012), whose study found that MIC and MBC of *E. coli* were 0.8 and 8 μg/mL, respectively. On the other hand, Sangani et al. (2015) reported that the MIC and MBC concentrations of ZnO-NPs were determined to be 158 and 325 μg/mL for 15 clinical isolates of *Pseudomonas aeruginosa* and 150 and 325 μg/mL for the reference strain, respectively. MIC results were determined to be 50 μg/mL for *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*, whereas it was determined to be 25 μg/mL for *Vibrio cholera* and *Clostridium botulinum*. The differences among these results may be due to the varying bacterial strains or to the differing methods of ZnO-NP preparation, which may affect the size and other properties of the nanoparticles.

**Table 1.** Zone of inhibition produced by zinc nitrate and zinc nanoparticles.

| Bacterial strains | Zinc nitrate | Bio-ZnNPs | Ciprofloxacin |
|------------------|-------------|-----------|--------------|
|                  | Concentration (μg/mL) | 20 | 40 | 60 | 160 | 320 | 320 |
| *B. subtilis*    | 9.0 ± 0.8 | 12.0 ± 0.6 | 15.0 ± 0.5 | 16.0 ± 0.6 | 22.0 ± 0.4 | 28.7 ± 0.3 | 32.0 ± 0.2 |
| *L. monocytogenes* | 10.0 ± 0.7 | 13.0 ± 0.6 | 17.0 ± 0.4 | 17.0 ± 0.5 | 22.7 ± 0.4 | 34.0 ± 0.2 | 36.0 ± 0.1 |
| *St. aureus*     | 8.0 ± 0.7 | 11.0 ± 0.7 | 14.0 ± 0.6 | 16.0 ± 0.6 | 19.0 ± 0.5 | 27.0 ± 0.3 | 31.0 ± 0.2 |
| *E. coli*        | 7.3 ± 0.9 | 10.0 ± 0.7 | 12.0 ± 0.9 | 14.0 ± 0.8 | 17.0 ± 0.5 | 25.0 ± 0.4 | 28.0 ± 0.5 |
| *K. pneumonia*   | 6.3 ± 0.9 | 9.0 ± 0.8 | 13.0 ± 0.7 | 14.0 ± 0.8 | 18.3 ± 0.5 | 26.7 ± 0.5 | 29.0 ± 0.5 |
| *S. enterica*    | 7.0 ± 0.9 | 10.0 ± 0.7 | 11.0 ± 0.8 | 13.0 ± 0.9 | 14.7 ± 0.4 | 24.7 ± 0.5 | 27.0 ± 0.6 |

Mean ± SD.
ZnO-NPs (Mishra et al. 2017). Particle size is important for obtaining high bacterial growth inhibition activity. The antimicrobial activity of nanoparticles depends on their manufacturing method, concentration, and size (Jiang et al. 2008), whereas activity was affected by nanoparticle size, which is controlled by processing parameters (Nagarajan and Rajagopalan 2008). Ghasemi and Jalal (2016) used clinical isolates of Acinetobacter baumannii resistant to multiple drugs (ciprofloxacin, ceftazidime, ampicillin, amikacin, cefalotin, and clavuloxacin) to study the inhibitory effects of ZnO-NPs. ZnO-NPs showed excellent inhibition of the MDR strain and also improved the antibacterial activity of ciprofloxacin and ceftazidime. Again, inhibitory effects were found to be concentration dependent, and maximum inhibition was observed when 0.25 mg/mL ZnO-NPs was combined with 8 μg/mL ciprofloxacin and 32 μg/mL ceftazidime. Thus, Acinetobacter baumannii isolates were otherwise resistant to both antibiotics showed significant inhibition when the antibiotics were used in combination with ZnO-NPs (Khatri et al. 2016).

**Antifungal activity of ZnO-NPs**

Nanoparticles possess good antifungal activity and though their exact mechanism of action is not known, the ranges of effectors are supposedly involved in fungal inhibition mediated by NPs. Apparently, disruption of membrane structure and the release of reactive oxygen species and hydrogen peroxide are mainly involved in the antimicrobial potential of ZnO-NPs (Sinha et al. 2011). Fungicidal action coupled with oxidative damage induced by ZnO-NPs most likely enhanced the inhibition of fungal growth. In addition, membrane disruption by ZnO-NPs could be another cause of the notable enhancement in their activity when in combination with NPs, as it increases the penetration and internalisation of the fungicide into the cells. ZnO-NPs have been individually investigated for their antimicrobial potential (Narendhran and Sivaraj 2016). The effects of ZnO-NPs on antifungal activity were found to be strictly dependent on the concentration of the ZnO-NPs used (Isaei et al. 2016). We preliminarily observed in the control treatments that a slight inhibition in the fungal growth of all the pathogenic fungal species was observed when they were treated with ZnNO₃ at 20 μg/mL as a positive control when compared with PDA plates used without ZnNO₃ addition as a negative control. Biologically synthesised ZnO-NPs exhibited good antifungal activity against the tested pathogenic fungi. All tested fungi showed a clear growth inhibition when increasing concentrations of ZnO-NPs are applied, but this inhibition was varied due to the ZnO-NPs concentrations used as well as the nature of the tested fungi. MIC values of biological ZnO-NPs were determined, and the results are presented in Table 3. MIC values of ZnO-NPs against the test fungi ranged between 35 and 85 μg/mL: the highest MIC level was recorded against Penicillium expansum at 85 μg/mL, while the lowest MIC level was 35 μg/mL recorded against Aspergillus niger (approximately a 60% decrease). The results obtained were found to be in accordance with available reports. Commercially available ZnO-NPs with the size of 70 ± 15 nm were found to significantly inhibit the growth of Botrytis cinerea and Penicillium expansum at concentrations greater than 3 mmol/L (approximately 244 μg/mL). Furthermore, Penicillium expansum was found to be more sensitive than Botrytis cinerea (He et al. 2011). In addition, a MIC value of 12.5 μg/mL was observed for Aspergillus niger, as reported by Singh and Nanda (12.5 μg/mL) (Singh and Nanda 2013). The mycelial growth of Rhizoctonia solani and Sclerotinia homoeocarpa were found to be substantially inhibited at ZnO-NP concentrations of 100 μg/mL.

### Table 2. The MIC and MBC of the zinc nanoparticles.

| Bacterial strains       | MIC (μg/ml) | MBC (μg/ml) |
|-------------------------|------------|-------------|
| Bacillus subtilis ATCC 6633 | 40         | 80          |
| Listeria monocytogenes ATCC 15313 | 35         | 70          |
| Staphylococcus aureus ATCC 8538 | 45         | 90          |
| Escherichia coli ATCC 8739 | 50         | 100         |
| Klebsiella pneumonia ATCC 27736 | 45         | 90          |
| Salmonella enterica ATCC 35664 | 50         | 100         |

Means with different small letter within column are significantly different. MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

### Table 3. the antifungal activity produced by zinc nitrate and zinc nanoparticles.

| Fungus Concentration (μg/mL) | Zinc nitrate MIC | Bio-ZnO-NPs MIC |
|-----------------------------|------------------|-----------------|
|                             | 20               | 20              |
| A. flavus                   | 10 ± 0.5         | 12 ± 0.2        |
| A. niger                    | 11 ± 0.4         | 13 ± 0.2        |
| F. oxysporium               | 9 ± 0.6          | 12 ± 0.2        |
| P. expansum                 | 8 ± 0.6          | 10 ± 0.3        |
| A. alternata                | 9 ± 0.6          | 11 ± 0.3        |

Means with different small letter within column are significantly different. MIC: minimum inhibitory concentration; Bio-ZnO-NPs: biological ZnO-NPs.
and ≥ 200 µg/mL, respectively (Li et al. 2017). These reports indicate that ZnO-NPs have excellent antimicrobial potential. Since the antimicrobial potential of NPs showed a correlation with NP size, shape, and concentration (Padmavathy and Vijayaraghavan 2008), analogous MIC values could be attributed to the similar shape and size range of NPs obtained through both green methods. The reason for the difference in the antifungal activity observed in different test microorganisms may be due to the difference in the microbial structure and thickness of its cell wall membrane. Moreover, antifungal activity was found to strongly depend on concentration. The presence of a high inhibition zone with nearly no growth occurrence against the tested strains clearly indicate that the mechanism of the fungicidal action of ZnO involves membrane disruption. These results agree with previously obtained results that demonstrated that ZnO nanoparticles can affect the viability of the pathogenic yeast, Candida albicans, and that this is a concentration-dependent effect (Shi et al. 2010).

ZnO-NPs possess an inhibitory effect on the growth of Aspergillus flavus and on its aflatoxins production. Aspergillus flavus is a deadly pathogen due to its production of highly carcinogenic aflatoxins, and there is a direct correlation between aflatoxin intake and liver cancer occurrence, with the International Agency for Research on Cancer listing aflatoxins as a highly carcinogen secondary metabolites. Outbreaks of aflatoxin contamination in food have drawn public attention to food safety. The aflatoxigenic A. flavus is found in most feed samples. Following this, ZnO-NPs have potential to be used in plant protection and used as a preservative for the safe storage of food products to prevent A. flavus contamination and aflatoxin poisoning (Kumari et al. 2019). Kumari et al. (2019) revealed that ZnO-NPs possess excellent capability to restrict A. flavus growth and aflatoxin production. Hence, this study may be a step towards the possible utilisation of ZnO-NPs in plant protection and as an antifungal agent especially against aflatoxins and/or aspergillosis, and may act as a preservative for the safe storage of food merchandize to prevent A. flavus contamination and aflatoxin-induced poisoning. ZnO-NPs displayed 92.25% inhibition of A. flavus growth and 100% inhibition of aflatoxin production at concentrations of 200 µl/mL and 150 µl/mL respectively. The result of anti-aflatoxigenic activity assays exhibited that ZnO-NPs can potentially inhibit aflatoxin biosynthesis from the toxigenic strain of Aspergillus flavus. ZnO-NPs inhibited 100% of aflatoxin biosynthesis at concentrations of 150 µl/mL and above. The growth of A. flavus and its aflatoxin biosynthesis were observed to decrease depending on the ZnO-NPs concentration. In addition, it was observed that the decrease in the mycelial weight of A. flavus leads to a reduction in aflatoxin production. Therefore, mycelial growth of this mould must be arrested below the threshold value in order to inhibit aflatoxin biosynthesis (Kumari et al. 2019).

**Growth performance**

The growth performance data of growing Japanese quails fed with the tested diets are shown in Table 4. Data revealed that a significant improvement in weight gain (WG) in groups fed with diet supplemented with biological nano zinc at levels of 0.1 or 0.2 g/kg of diet. The body weights at 21 days of age were quadratically significant (p ≤ .0001), improving by

| Items                  | Biological nano zinc levels (g/kg diet) | SEMa | p Valueb          |
|------------------------|----------------------------------------|------|-------------------|
| Body weight (g)        |                                        |      |                   |
| 1 week                 | 26.88 26.90 27.03 26.87 27.00          | 0.182| .7303 .9174       |
| 3 weeks                | 90.80 100.19 97.97 95.94 91.32         | 0.965| .3358 <.0001      |
| 5 weeks                | 177.09 189.68 203.39 186.55 178.50    | 2.062| .9639 <.0001      |
| Body weight gain (g / day) |                                    |      |                   |
| 1–3 weeks              | 4.57 5.24 5.07 4.93 4.59              | 0.062| .2319 <.0001      |
| 3–5 weeks              | 6.16 6.39 7.53 6.47 6.23              | 0.195| .7614 .0025       |
| 1–5 weeks              | 5.36 5.81 6.30 5.70 5.41              | 0.076| .9581 <.0001      |
| Feed intake (g / day)  |                                        |      |                   |
| 1–3 weeks              | 14.40 14.00 14.03 14.19 13.82          | 0.261| .3186 .8655       |
| 3–5 weeks              | 23.04 19.74 22.26 22.05 21.50          | 0.236| .4279 .0320       |
| 1–5 weeks              | 18.72 16.87 18.14 18.12 17.66          | 0.204| .2351 .1037       |
| Feed conversion ratio (g / g) |                                    |      |                   |
| 1–3 weeks              | 3.15 2.67 2.77 2.88 3.01              | 0.047| .6543 .0002       |
| 3–5 weeks              | 3.74 3.10 2.96 3.41 3.46              | 0.066| .3759 <.0001      |
| 1–5 weeks              | 3.49 2.90 2.88 3.18 3.26              | 0.035| .2582 <.0001      |

aStandard error means.
bLinear and quadratic effects.
10.34 and 7.89% by increasing the supplementation of biological nano zinc from 0.1 to 0.2 g/kg diet, respectively, when compared with the control. At the end of the experimental period, there was a significant ($p \leq .0001$) the BW of birds fed with a diet supplemented with biological nano zinc at levels of 0.1 and 0.5 g/kg diet improved by 7.11 and 14.85%, respectively, compared to the control group. The same trend was observed for weight gain. For the entire experimental period, birds fed with a diet supplemented with nano zinc at a concentration of 0.2 g/kg significantly had ($p \leq .0001$) the highest WG followed by those fed with diets supplemented with 0.1 g/kg followed by those fed with 0.3 g/kg diet when compared to control.

There were no significant differences in feed intake (FI) between the different experimental groups during the trial period, except in the period of 3–5 weeks of age. In that period, a significant ($p = .0320$) decrease in the FI occurs as a result biological nano zinc addition. Birds fed with diet supplemented with a nano zinc concentration of 0.1 g/kg feed corresponded to the lowest amount of feed consumed, and was lower by 14.32%, followed by those fed on 0.4 g/kg diet, which had FI lower by 6.68% compared to the control group.

Based on this, a significant improvement in the feed conversion ratio (FCR) as a result of feeding with experimental diets was observed. Whereas at the end of the experimental period, birds fed with biological nano zinc at a dose of 0.2 g/kg diet significantly achieved ($p \leq .0001$) the highest FCR, being better by 17.47% compared to control; this is followed by those fed with a nano zinc concentration of 0.1 g/kg diet, which was better by 16.90%.

Biological nano zinc supplementation promotes traits including BW, WG, FI, and FCR at the concentrations of 0.2 or 0.3 g/kg diet. The positive effect of ZnO-NPs supplementation on growth may be due the important role of Zn in the overall performance and physiological process of poultry, as it is the main component of a large number of enzymes known as metalloenzymes, which are involved in metabolism of energy, nucleic acids, and protein (Torres and Korver 2018; Attia et al. 2019). In addition, nanoparticles may have caused faster diffusion through the gastro-intestinal tract GIT mucus to reach cells of the intestinal lining, followed by absorption through the GIT barrier to reach the blood (El-Rayes et al. 2019). Moreover, this effect can be attributed to the improvement in intestine development and increase in the relative weight of digestive organs, as previously reported (Mohammadi et al. 2015) or due to the upregulation of growth-related genes. The current results are compatible with Fathi (2016) who demonstrated that appropriate levels of nano-ZnO can promote BW, WG, and FCR. However, higher nano-ZnO concentrations inhibited body weight gain.

### Hematological parameters

Table 5 represents the hematological parameters of growing Japanese quail as affected by biological nano zinc.

| Biological nano zinc levels (g/kg diet) | 0 | 0.1 | 0.2 | 0.3 | 0.4 | SEM$^a$ | $p$ Value$^b$ |
|--------------------------------------|---|-----|-----|-----|-----|--------|-------------|
|                         |   |     |     |     |     |        |             |
| Carcase %                | 75.5 | 75.98 | 78.08 | 79.32 | 75.66 | 1.647 | 1.545 | 0.1832 |
| Liver %                  | 2.53 | 2.58 | 2.05 | 2.24 | 2.67 | 0.139 | 0.8834 | 0.0226 |
| Gizzard %                | 2.38 | 2.4 | 2.02 | 2.38 | 2.02 | 0.118 | 1.342 | .9762 |
| Heart %                  | 1.06 | 0.99 | 1.08 | 1.18 | 1.01 | 0.086 | 0.7177 | .6027 |
| Giblets %                | 5.97 | 5.97 | 5.15 | 5.79 | 5.71 | 0.206 | 0.3380 | .1506 |
| Dressing %               | 81.47 | 81.95 | 83.23 | 85.11 | 81.36 | 1.726 | 1.6093 | .2615 |

$^a$Standard error means.

$^b$Linear and quadratic effects.
blood cells (WBCs), red blood cells (RBCs), and haematocrit (HCT). In addition, the group fed with 0.4 g/kg diet had the highest mean corpuscular volume (MCV) when compared to control. However, mean corpuscular haemoglobin (MCH) decreased due to a decrease in biological nano zinc supplementation levels from 0.4 to 0.1 g/kg diet compared to the control. Current results are compatible with those observed by Aliarabi et al. (2015), who found that hematological parameters that were decreased (p < .05) by Zn deficiency included Hb, total erythrocyte count, and packed cell volume, with the proportion being dose-dependent.

### Table 6. Hematological parameters of growing Japanese quail as affected by biological nano zinc.

| Items          | Biological nano zinc levels (g/kg diet) | SEMa | p Valueb     |
|----------------|-----------------------------------------|------|--------------|
|                | 0    | 0.1  | 0.2  | 0.3  | 0.4  |      |      |
| WBCs (103/μl)c | 179.01 | 291.96 | 201.73 | 246.19 | 182.29 | 10.409 | .5439 | .0141 |
| LYM (%)        | 0.95  | 0.92  | 0.95  | 0.93  | 0.95  | 0.013 | .8384 | .5416 |
| MID (%)        | 0.05  | 0.07  | 0.05  | 0.07  | 0.05  | 0.012 | .9099 | .5166 |
| GRA (%)        | 0.004 | 0.005 | 0.002 | 0.006 | 0.002 | 0.001 | .4332 | .6553 |
| RBCs (106/μl)  | 1.43  | 2.46  | 1.91  | 2.08  | 1.59  | 0.154 | .9347 | .0035 |
| HGB (g/dL)     | 11.7  | 13.33 | 10.47 | 12.63 | 11.27 | 0.915 | .6103 | .7894 |
| HCT (%)        | 16.93 | 31.47 | 24.30 | 28.23 | 21.43 | 1.847 | .4125 | .0027 |
| MCV (μm3)      | 118.80 | 127.67 | 126.53 | 130.93 | 134.27 | 2.067 | .0008 | .5303 |
| MCH (pg)       | 81.67 | 54.00 | 54.60 | 60.83 | 71.03 | 2.038 | .0552 | <.0001 |
| PLT (103/μl)   | 16.67 | 24.33 | 18.00 | 15.00 | 18.33 | 3.494 | .6501 | .7327 |

aStandard error means.
bLinear and quadratic effects.
cWBCs: white blood cells; LYM: lymphocytes; MID: mid-range; GRA: granulocytes; RBCs: red blood cells; HGB: haemoglobin; HCT: haematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; PLT: Platelet count.

### Table 7. Blood constituents of growing Japanese quail as affected by biological nano zinc.

| Items          | Biological nano zinc levels (g/kg diet) | SEMa | p Valueb     |
|----------------|-----------------------------------------|------|--------------|
|                | 0    | 0.1  | 0.2  | 0.3  | 0.4  |      |      |
| TP (g/dL)c     | 3.38  | 3.99  | 3.30  | 3.29  | 3.69  | 0.092 | .8255 | .5237 |
| ALB (g/dL)     | 1.24  | 1.36  | 1.25  | 1.19  | 1.28  | 0.030 | .3759 | .9189 |
| GLOB (g/dL)    | 2.14  | 2.63  | 2.05  | 2.10  | 2.40  | 0.062 | .8792 | .3546 |
| A/G (%)        | 0.58  | 0.52  | 0.62  | 0.57  | 0.53  | 0.010 | .2505 | .0820 |
| AST (IU/L)     | 185   | 150   | 170   | 173   | 207   | 3.289 | .0002 | <.0001 |
| ALT (IU/L)     | 9.96  | 7.48  | 8.79  | 9.30  | 14.52 | 0.744 | .0151 | .0006 |
| LDH (IU/L)     | 154   | 153   | 143   | 163   | 189   | 5.652 | .0018 | .0035 |
| Creatinine (mg/dL) | 0.33  | 0.32  | 0.41  | 0.35  | 0.36  | 0.022 | .3065 | .2260 |
| Urea (mg/dL)   | 7.28  | 6.99  | 6.96  | 6.69  | 6.88  | 0.054 | .0002 | .0105 |
| TC (mg/dL)     | 233.35 | 251.85 | 237.65 | 199.50 | 257.10 | 4.786 | .7833 | .0234 |
| TG (mg/dL)     | 227.50 | 219.50 | 215.35 | 225.95 | 236.15 | 6.241 | .2756 | .0621 |
| HDL (mg/dL)    | 37.27  | 50.82  | 55.33  | 45.93  | 39.26  | 2.153 | .8981 | <.0001 |
| LDL (mg/dL)    | 150.58 | 173.13 | 139.25 | 108.39 | 170.61 | 1.386 | .5302 | <.0001 |
| VLDL (mg/dL)   | 45.50  | 43.90  | 43.07  | 45.19  | 47.23  | 1.248 | .2756 | .0621 |
| SOD (U/ml)c    | 0.21  | 0.32  | 0.37  | 0.24  | 0.26  | 0.026 | .8099 | .0100 |
| MDA (nmol/mL)  | 0.36  | 0.20  | 0.18  | 0.17  | 0.20  | 0.023 | .0027 | .0053 |
| GPX (mg/dL)    | 0.22  | 0.33  | 0.39  | 0.23  | 0.23  | 0.024 | .4837 | .0098 |
| IgG (mg/dL)    | 0.96  | 1.26  | 1.56  | 1.52  | 0.95  | 0.065 | .3373 | <.0001 |
| IgM (mg/dL)    | 0.65  | 0.80  | 1.26  | 1.39  | 0.64  | 0.077 | .0570 | <.0001 |
| Zinc (mg/dL)   | 57.50  | 65.00  | 91.50  | 66.50  | 76.50  | 3.233 | .0038 | .0039 |

aStandard error means.
bLinear and quadratic effects.
cTP: total protein; Alb: albumin; GLOB: globulin; A/G: albumin/ globulin ratio; AST: aspartate aminotransferase and ALT: alanine aminotransferase. LDH: lactate dehydrogenase.
TC: total cholesterol; TG: triglycerides; HDL: high density lipoprotein; LDL: low density lipoprotein; SOD: superoxide dismutase; GPX: glutathione peroxidase; MDA: malondialdehyde; IgG: immunoglobulin G; IgM: immunoglobulin M; VLDL: very-low-density lipoprotein.

### Blood constituents

Liver and kidney function of growing Japanese quails as affected by dietary treatments are illustrated in Table 7. Results showed that plasma total protein and albumin as well as creatinine, were not linearly nor quadratically influenced by biological nano zinc supplementation levels. On the other hand, the dietary supplementation of biological nano zinc, especially at low levels (0.1–0.3 g/kg diet), demonstrated a positive impact on the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) and on urea concentration. However, a high level of biological nano zinc (0.4 g/kg diet)
diet) resulted in negative effects for the same indices except for urea concentration.

The effects of dietary biological nano zinc on liver profile parameters including TC, TG, HDL, LDL, and VLDL in growing Japanese quails, as affected by dietary treatments, are illustrated in Table 7. Liver profile parameters were not statistically (p > .05) influenced by dietary biological nano zinc with the exception of TC, HDL, and LDL. Compared to the control group, the supplementation of diets with biological nano zinc at levels of 0.2 and 0.3 g/kg diet significantly (p < .001) decreased TC and LDL levels in the serum of growing Japanese quail. In addition, birds that were fed with diet supplemented with biological nano zinc at dose of 0.2 g/kg diet significantly (p < .0001) had the highest value for HDL, followed by those that received 0.1 g/kg and then those fed with 0.3 g/kg, respectively.

The impact of dietary biological nano zinc on antioxidants and on the immunity status of the growing Japanese quail, as affected by dietary treatments are presented in Table 7. Dietary biological Zn-NP levels significantly (p < .001) affected antioxidant indices in serum, including superoxide dismutase SOD, malondialdehyde MDA, glutathione peroxidase GPX, and zinc concentration. The activity and concentration of previous indices were improved with biological Zn-NPs supplementation compared to the control group. Moreover, dietary supplementation of biological Zn-NPs at the dosages from 0.1 up to 0.3 g/kg diet exhibited a positive impact on Immunoglobulin G (IgG) and Immunoglobulin M (IgM) levels.

Blood biochemistry parameters are important markers of physiological status (El-Kholy et al. 2017; Alagawany et al. 2018; Farag and Alagawany 2018). The blood parameters of the treated groups varied significantly in terms of ALT, AST, LDH, urea, TC, HDL, LDL, and serum Zn content, as their values increased with increasing zinc level. Current results are consistent with Fathi (2016), who reported that 20 mg/kg nano-ZnO tended to increase serum cholesterol. Moreover, a change of cholesterol levels in the blood plasma may be due to zinc’s role in enzymatic actions, in that zinc forms an integral part of several enzymes (metalloenzymes) that are severed in lipid digestion and absorption (Hazim et al. 2011; Abd El-Hack et al. 2017a, 2017b, 2020). Additionally, zinc-deficient diets are accompanied by decreased plasma TC, LDL, HDL, and TG concentrations. This can be due to the diminished uptake of dietary lipids, as well as due to a reduction of fat and calorie intake. Furthermore, Roberson and Edwards (1994) reported that there is a significant increase in serum HDL and TC for the zinc supplemented group. These researchers suggested that increased levels of HDL and cholesterol are probably due to an improvement in calorie and fat intake after zinc supplementation.

Zinc is a fundamental component in SOD, and there a positive correlation between dietary Zn levels and SOD activity was observed. It has been demonstrated that SOD is involved in cellular scavenging of free radicals and ROS (Prasad 2008; Abd El-Hack et al. 2018a, 2018b). The current results are compatible with those previously reported by Duzguner and Kaya (2007). MDA is an important index for lipid peroxidation and oxidative damage caused by ROS. Our data confirm the hypothesis that appropriate concentrations of nano-ZnO stimulate SOD activity, and enhanced SOD will suppress ROS generation, thus decreasing MDA levels. Our findings are consistent with those from a previous report (Duzguner and Kaya 2007; Attia et al. 2013).

### Caecal microbial count

Caecal microbiota data of growing Japanese quails as affected by dietary treatments are presented in Table 8. Means of microbiological count include total bacterial count (TBC), total yeasts and molds count (TYMC), *E. coli*, coliform, lactic acid bacteria, *Enterococcus* spp., and *Salmonella* spp., and these were linearly

| Items                     | Biological nano zinc levels (g/kg diet) | p Value<sup>b</sup> |
|---------------------------|----------------------------------------|--------------------|
|                           | 0           | 0.1       | 0.2       | 0.3       | 0.4       | SEM<sup>a</sup> | linear | quadratic |
| TBC                       | 5.88        | 6.05      | 5.11      | 6.05      | 5.30      | 0.013       | <.0001 | .4003     |
| TYMC                      | 5.59        | 5.83      | 4.96      | 5.77      | 5.14      | 0.041       | .0011 | .6472     |
| *E. coli*                 | 5.79        | 5.96      | 5.06      | 6.01      | 5.25      | 0.037       | <.0001 | .9288     |
| Lactic acid bacteria      | 5.74        | 5.61      | 5.02      | 5.87      | 5.23      | 0.047       | .0022  | .0924     |
| *Enterococcus*            | 5.73        | 6.04      | 5.86      | 6.00      | 5.29      | 0.042       | <.0001 | <.0001    |
| *Salmonella*              | 5.75        | 5.87      | 4.95      | 5.82      | 5.17      | 0.046       | <.0001 | .2268     |

<sup>a</sup>Standard error means.<br><sup>b</sup>Linear and quadratic effects.<br><sup>c</sup>TBC: total bacterial count; TYMC: total yeasts and molds count.
(p < .0001) affected in all supplemented groups compared to control group. In general, the dietary supplementation of biological nano zinc at a dose of 0.1 g/kg diet led to a significant increase in all microbiological counts except for *E. coli* and *Enterococcus* spp. On the other hand, adding biological nano zinc at doses of either 0.2 or 0.4 g/kg diet resulted in a significant (p < .0001) decrease in the number of microbial populations except for *Enterococcus* for the level of 0.4 g/kg diet. Moreover, dietary supplementation of biological nano zinc at a level of 0.3 g/kg diet led to a significant (p < .0001) increase in all microbiological counts, except for *Salmonella* spp., which was reduced.

Zinc oxide nanoparticles are externally used for treatment of mild bacterial infections (Fontecha-Umaña et al. 2020). It is known that ZnO-NPs are antibacterial and inhibit the growth of microorganisms by permeating the cell membrane. Oxidative stress damages lipids, carbohydrates, proteins, and DNA (Tiwari et al. 2018). Our results are in accordance with those reported by Soren et al. (2018) who found that ZnO in aqueous suspensions had stronger bactericidal activity against the Gram-negative bacterium *E. coli* than against the Gram-positive bacterium *S. aureus*.

**Conclusions**

From the obtained results, it could be concluded that biologically synthesised ZnO-NPs exhibited a good antifungal activity against the tested pathogenic fungi. All tested fungi showed a clear growth inhibition when increasing concentrations of ZnO-NPs are applied, but this inhibition was varied due to the ZnO-NPs concentrations used as well as the nature of the tested fungi. ZnO-NPs synthesized by Bacillus subtilis AM12 supernatant showed an excellent inhibition of the MDR strains as compared to ciprofloxacin. Also, dietary supplementation of biological ZnO-NPs at 0.2 g/kg diet had a positive effect on the performance and physiological status of growing Japanese quails.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Ethical approval**

Animal care and maintenance were performed in accordance with the guidelines of the Egyptian Research Ethics Committee and the guidelines specified in the Guide for the Care and Use of Laboratory Animals (2011).

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