Efficacy of using zinc oxide nanoparticle as a substitute to antibiotic growth promoter and zinc sulphate for growth performance, antioxidant capacity, immunity and intestinal barrier function in broilers

Jiaqi Zhang, Zhihua Li, Caïyun Yu, Huijuan Liu, Binbin Zhou, Xuhui Zhang, Tian Wang and Chao Wang

College of Animal Science and Technology, National Experimental Teaching Demonstration Centre of Animal Science, Nanjing Agricultural University, Nanjing, China; Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, Nanjing, Jiangsu, China

ABSTRACT
Dietary zinc oxide nanoparticle (ZnO NP) possessing multiple biological activities might be a potential substitute for the combination of ZnSO4 and antibiotic growth promoter in broiler diets. Therefore, this study was conducted to investigate the efficacy of dietary ZnO NP as a substitute to the combination of ZnSO4 and xanthomycin for growth, antioxidant capacity, immunity, intestinal barrier and liver function in broilers. Arbor Acres chicks (n = 320) were assigned to 5 treatments with 8 replicates. Birds received a basal diet supplemented with 80 mg/kg ZnSO4 plus 5 mg/kg xanthomycin (ZnSO4 + Xanthomycin) or 0 (negative control, NC), 40, 80, and 160 mg/kg ZnO NP for 42 days. The average daily gain, average daily feed intake and feed-to-gain ratio showed dose-dependent increases with the increasing level of dietary ZnO NP during the 21–42 day and 1–42 day stages. The final body weight (42 d) and serum concentrations of insulin-like growth factor 1 and growth hormone increased linearly with the increasing level of dietary ZnO NP. In addition, 80 mg/kg ZnO NP increased the serum concentrations of IgA, IgG, IgM, and interleukin-10 and peroxidase activity, the jejunal mucosal villus height, villus width and goblet cell numbers. Dietary 80 or 160 mg/kg ZnO NP significantly altered mRNA abundances of genes related to antioxidant status, intestinal barrier and immunity in the jejunal mucosa. These results indicated that dietary supplementation with 40–160 mg/kg ZnO NP caused no obvious negative effects on liver function, but effectively improved growth performance, intestinal barrier function, immunity and antioxidant capacity.

HIGHLIGHTS
- Dietary 40–160 mg/kg zinc oxide nanoparticle (ZnO NP) improved growth performance and enhanced immunity of broilers without obvious negative effects on liver function.
- Dietary 40–160 mg/kg ZnO NP improved intestinal barrier and intestinal morphology, and enhanced antioxidant capacity via Nrf2/HO-1 pathway.
- Doses of 40–80 mg/kg ZnO NP were suggested to alternate the combination of 80 mg/kg ZnSO4 and 5 mg/kg xanthomycin in diets of broilers.

Introduction
The poultry industry has become the fastest growing industry and the biggest contributor to gross domestic product in many countries (Parker 2016; Bobkov and Zbinden 2018). The chicken is an exemplar of efficient intensive animal agriculture and provides many valuable animal protein products (Tizard et al. 2019). However, this efficiency and intensive production comes with a number of challenges, such as the prevalence of stresses and increased risk of diseases. In the past, antibiotics were widely used as growth promoters in animal feeds to enhance immune system and improve growth and production (Barton 2000; Vondruskova et al. 2010). However, antimicrobial resistance and the possible transfer to human microbiota caused by the abuse of antibiotics have aroused global concerns (Barton 2000; Castanon 2007). Since 2006, antibiotics have been banned as feed growth promoters in European Union countries (Castanon 2007). China has also imposed a ban on the use of...
antibiotics in animal feed since 1 January 2021 (Announcement No. 194, 2019). Consequently, the demand is growing fast for dietary antibiotic alternatives to reduce the challenges in intensive animal and poultry production.

Zinc, a crucial trace element, is an integral component of over 300 enzymes and is closely related to animal immune function and gut development (Han and Thacker 2009; Vondruskova et al. 2010). The highly bioavailable form, ZnSO₄, is commonly used in poultry diets to meet zinc requirements for growth (Leeson and Caston 2008; Mwangi et al. 2017). However, the ban on antibiotics has created difficulties in achieving the goal for the rapid and healthy growth only with the traditional ZnSO₄ in broiler diets alone. Zinc in nanoscale metal form has recently been suggested as a novel mineral feed additive in poultry diets (Sizova et al. 2018). Zinc oxide nanoparticle (ZnO NP) shows high bioavailability, which is as bioavailable as the ionic zinc and can be used as an alternative to organic and inorganic zinc forms in animal feeds (Antoine et al. 2016; Swain et al. 2016; Mahmoud et al. 2021). Therefore, we speculated that ZnO NP might be a potential substitute for the combination of ZnSO₄ and antibiotics in broiler diets, which might be widely used in China and several other countries to promote growth and health in practical production. Previous studies have shown that xanthomycin is stable and has multiple properties, such as promoting animal growth by regulating intestinal flora and improving the digestion of energy and protein in the feed (Min et al. 2016; Yuan et al. 2018; He et al. 2019). It was widely used as an antibiotic growth promoter in livestock animals, especially in broiler chickens in China before the ban on dietary antibiotics. Taken together, this study was conducted to investigate the efficacy of ZnO NP as a substitute to the combination of ZnSO₄ and xanthomycin for growth performance, antioxidant status, immune and intestinal barrier and liver function in broilers.

Materials and methods

Characterisation of ZnO NP

The ZnO NP (99.5%), a white powdery solid, was provided by Zhangjiagang Bonded Area Hualu Nanometer Material Co., Ltd. (Jiangsu, China). The primary particle size of ZnO NP was determined by transmission electron microscope (TEM) at 80 kV (H-7500, Hitachi High-Technologies Corporation, Japan).

Experimental design and diets

This study was approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) and was conducted under the corresponding supervision (Permit number: SYXK-2019-00085).

A total of 320 1-day-old healthy male Arbor Acres chicks, each weighing about 38.0 g, were obtained from a local commercial hatchery. The chicks were divided into 5 groups with 8 replicates cages (150 cm × 100 cm × 80 cm) of 8 birds per cage. All birds had free access to feed and water in this 42 d experiment. The environmental temperature was controlled at 33°C to 34°C for the first 4 days and gradually decreased to 26–27°C during days 5–21; it was subsequently kept at 22°C to 24°C until 42 days of age. The lighting program was 22 and 23 h light per day during days 1–21 and days 22–42, respectively. Broilers received a basal diet supplemented with 80 mg/kg Zn from ZnSO₄ plus 5 mg/kg xanthomycin (ZnSO₄ + Xanthomycin group), or 0 (negative control, NC), 40, 80, 160 mg/kg Zn from ZnO NP. The ZnSO₄ (34.5 g/kg of product) and xanthomycin (80 g/kg of product) were provided by Shandong Shengli Co., Ltd. (Shandong, China). The ingredients and chemical compositions of the 5 experimental diets are listed in Table 1. The contents of dry matter (method 934.01), ash (method 925.16), crude protein (kjeldahl N × 6.25, method 981.10), ether extract (method 920.39), calcium (method 927.02) and phosphorus (method 965.17) were determined (AOAC 2000). The neutral detergent fibre (aNDFom) and acid detergent fibre (ADFom) contents were determined using the procedure reported by Van Soest et al. (1991). The gross energy was obtained with a calorimeter provided by Hebi Tianyu Instrument Manufacturing Co., Ltd (Henan, China). The amino acid contents were determined by the amino acid analyser (LA8080, Hitachi...
High-Technologies Corporation, Tokyo, Japan) using method 994.12 (AOAC 1995). The zinc content was determined by treating feed samples at 550 °C for 4 h, dissolving the residual ash in 6 mol/L HCl, filtering and diluting to an optimal level for determination of the zinc content by flame atomic absorption spectrometry (Z-2000, Hitachi High-Technologies Corporation, Tokyo, Japan).

Sample collection, growth performance and organ index

At 1, 21 and 42 days of the experiment, the body weight (BW) and the feed intake of broilers were obtained to calculate the feed-to-gain ratio (F:G), average daily gain (ADG) and average daily feed intake (ADFI). At 42 d, one bird (with a BW close to mean BW) per replicate was weighed, euthanized by electrical stunning and exsanguinated after fasting 12 h. After that, the blood, jejunum, liver, thymus, spleen and bursa of fabricius samples were collected rapidly. The blood was centrifuged (3,000 g, 15 min, 4 °C) to separate the serum. The liver, spleen, thymus and bursa of fabricius samples were weighed to calculate the organ index (organ index (g/100 g) = organ weight (g)/body weight (g)/100). Samples for biochemical assays were stored at −80 °C.

Determination of serum parameters

Concentrations of serum immunoglobulin A (IgA, Catalogue no. ANG-E32004C), immunoglobulin G (IgG, Catalogue no. ANG-E32009C), immunoglobulin M (IgM, Catalogue no. ANG-E32005C), interleukin 10 (IL-10, Catalogue no. ANG-E32011C), tumour necrosis factor α (TNF-α, Catalogue no. ANG-E32030C), insulin-like growth factor 1 (IGF-1, Catalogue no. ANG-E32048C) and growth hormone (GH, Catalogue no. ANG-E32049C) were measured with corresponding ELISA assay kits from Nanjing Aoqing Co., Ltd (Nanjing, China), according to the manufacturer’s instructions. These parameters were determined for further evaluation of immune system and growth-related hormones in the broilers.

Table 1. Ingredients and chemical compositions of diets (as-fed basis).

| Item | Starter (days 1–21) | Finisher (days 22–42) |
|------|-------------------|-----------------------|
|      | ZnSO₄ + Xanthomycin³ | ZnO NP (mg/kg) | ZnSO₄ + Xanthomycin | ZnO NP (mg/kg) |
|      | 0 (NC) 40 80 160 | 0 (NC) 40 80 160 |
| Corn | 570.1 570.1 570.1 570.1 | 570.1 570.1 570.1 570.1 |
| Soybean | 315 315 315 315 | 315 315 315 315 |
| Corn gluten meal | 34 34 34 34 | 34 34 34 34 |
| Soy oil | 31 31 31 31 | 31 31 31 31 |
| Dicalcium phosphate | 20 20 20 20 | 20 20 20 20 |
| Limestone | 12 12 12 12 | 12 12 12 12 |
| L-Lysine | 3.4 3.4 3.4 3.4 | 3.4 3.4 3.4 3.4 |
| DL-Methionine | 1.5 1.5 1.5 1.5 | 1.5 1.5 1.5 1.5 |
| Sodium chloride | 3 3 3 3 | 3 3 3 3 |
| Premix⁴ | 10 10 10 10 | 10 10 10 10 |
| Total | 1,000 1,000 1,000 1,000 | 1,000 1,000 1,000 1,000 |
| Chemical analysis (g/kg) | | |
| Dry Matter | 902.61 903.63 903.92 903.21 | 901.65 905.26 906.29 906.33 |
| Crude protein | 216.40 216.54 217.28 216.98 | 219.27 192.15 192.33 192.15 |
| Crude ash | 62.17 62.65 63.09 62.68 | 61.76 65.93 65.58 66.91 |
| Ether extract | 53.05 53.92 52.97 53.64 | 53.19 74.66 75.26 74.87 |
| aNDFom | 144.88 145.25 143.22 144.45 | 143.45 162.54 161.58 161.70 |
| ADFom | 62.02 62.68 63.33 63.07 | 62.81 65.22 65.17 64.97 |
| Methionine | 5.00 5.02 5.07 5.09 | 5.08 4.17 4.11 4.17 |
| Lysine | 12.30 12.21 12.29 12.24 | 12.22 10.51 10.45 10.49 |
| Cystine | 3.65 3.62 3.62 3.66 | 3.64 3.45 3.49 3.46 |
| Calcium | 12.41 12.39 12.93 12.39 | 12.25 11.64 12.45 12.70 |
| Phosphorus | 7.03 6.87 7.00 7.00 | 6.97 6.22 6.24 6.28 |
| Zn (mg/kg) | 119.88 40.78 78.31 123.48 | 203.38 124.52 46.88 89.12 |
| Gross energy (kcal/kg) | 4,643.77 4,641.38 4,679.62 4,674.84 | 4,684.40 4,756.10 4,794.34 4,732.20 |
| ME (kcal/kg) | 2,997.06 2,997.06 2,997.06 2,997.06 | 2,997.06 3,116.56 3,116.56 3,116.56 |

¹ADFom, acid detergent fibre expressed exclusive of residual ash; DM, dry matter; ME, metabolisable energy; NC, negative control; aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; ZnO NP, zinc oxide nanoparticle.
²ZnSO₄ + Xanthomycin group, basal diet supplemented with 80 mg/kg ZnSO₄ and 5 mg/kg xanthomycin; NC group, basal diet; 40, 80 and 160 mg/kg ZnO NP group, basal diet supplemented with 40, 80 and 160 mg/kg ZnO NP, respectively.
³Premix provided for each kilogram diet: vitamin E, 30 IU; vitamin D₃, 3,000 IU; vitamin A, 10,000 IU; riboflavin, 8 mg; vitamin B₁₂, 0.013 mg; thiamine, 2.2 mg; menadione, 1.3 mg; choline chloride, 600 mg; nicotinamide, 40 mg; calcium pantothenate, 10 mg; biotin, 0.04 mg; pyridoxine-HCl, 4 mg; folic acid, 1 mg; Fe, 80 mg; Se, 0.3 mg; Cu, 8.0 mg; iodine, 1.1 mg; Mn, 110 mg.
The intestinal barrier function was examined in the broilers by measuring the levels of D-lactate (Catalogue no. ANG-E32105C) and endotoxin (Catalogue no. ANG-E32028C) using corresponding assay kits (Nanjing Aoqing Co., Ltd, Nanjing, China). The activity of diamine oxidase (DAO, Catalogue no. AA88-2) was measured with a kit from Nanjing Jiancheng Institute (Nanjing, China).

Liver function was evaluated in the broilers by determining the activities of serum glutamic-pyruvic transaminase (GPT, Catalogue no. C009-2-1), glutamic oxaloacetic transaminase (GOT, Catalogue no. C010-2-1) and alkaline phosphatase (AKP, Catalogue no. A059-2-2) with kits supplied by Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

Antioxidant capacity of the broilers was evaluated by determining the serum concentrations of protein carbonyl (PC, Catalogue no. A087) and malondialdehyde (MDA, Catalogue no. A003-1-2), and activities of superoxide dismutase (SOD, Catalogue no. A001-1-1), peroxidase (POD, Catalogue no. A084-2-1) and the total antioxidant capacity (T-AOC, Catalogue no. A015-2-1) using the respective detection kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

Histological analysis
The jejunum histology of broilers was analysed by periodic acid-Schiff (PAS) and haematoxylin and eosin (H&E) staining. The jejunum was removed from the digestive tract and fixed in paraformaldehyde. The fixed jejunum was cleared, dehydrated, paraffin embedded, sectioned, stained with H&E and examined by light microscopy. Crypt depth, villous height and villous width were obtained with Image Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, USA). PAS-stained tissues were used to determine the number of goblet cells from one intact villus by counting 100 absorptive epithelial cells. Three intact villi per field were selected, and two fields were examined for each broiler.

The liver samples were taken from the same location in each bird for analysis of the histomorphology via H&E staining, as described previously (Chen et al. 2018). Briefly, after fixation, clearing, dehydration and paraffin embedding, the liver samples were sectioned at 5 μm thickness, stained with H&E, and observed using an optical binocular microscope (Olympus BX5; Olympus Optical Co. Ltd, Tokyo, Japan) equipped with a digital camera (Nikon HS550L; Nikon, Tokyo, Japan).

Analysis of mRNA expression
The extracted total RNA (from the jejunal mucosa) with Trizol reagent (Vazyme Biotech Co., Ltd, Nanjing, China, Catalogue no. R401-01) was quantified based on the light absorption with a Nanodrop ND-2000c spectrophotometer (the ratio of absorption at 260/280 nm and 260/230 nm in a range from 1.90 to 2.05) and tested by agarose gel electrophoresis. The quantified RNA (1 μg) was used to obtain the cDNA with the reverse transcription kit (TaKaRa Biotechnology Co. Ltd, Dalian, China, Catalogue no. 2690A) according to the manufacturer's instructions. The cDNA was further amplified with the TB Green Premix Ex Taq (Tli RNaseH Plus) kit (TaKaRa, Dalian, Liaoning, China) by the real-time polymerase chain reaction (RT-PCR) with the StepOne Plus ABI Prism Sequence Detection system (Applied Biosystems, Foster City, CA, USA). Relative mRNA expression was normalised to the NC group with the 2^(-ΔΔCt) method (Livak and Schmittgen, 2001). The specific primers used for genes related to functions of intestinal barrier [claudin-2, zonula occludens-1 (ZO-1), mucin2 and occludin], antioxidant capacity [haem oxygenase-1 (HO-1), nuclear factor erythroid-2-related (Nrf2), kelch-like ECH associated protein 1 (Keap1), catalase (CAT), SOD, glutathione peroxidase (GSH-Px) and NAD(P)H dehydrogenase, quinone 1 (NQ01)] and inflammation [interferon-γ (IFN-γ), interleukin-1β (IL-1β), toll-like receptor 4 (TLR4), transforming growth factor-beta-activated kinase-1 (TAK1), myeloid differentiation primary response protein 88 (MYD88), interleukin-2 (IL-2) and adenosine monophosphate-activated protein kinase (AMPK)] are listed in Table 2. The primers were well used in previous studies (Chen et al. 2016; Shen et al. 2019) or designed and synthesised by a commercial company (Invitrogen Biotech Co. Ltd., Shanghai, China). The mRNA expression level of each target gene was normalised to the level of β-actin.

Statistical analysis
Data were processed with the SPSS statistical package (version 28.0 for Windows, SPSS, Inc., Chicago, IL, USA). One-way analysis of variation (ANOVA) with Tukey's test was used to evaluate the differences among the 5 groups. For the polynomial trend analysis, 4 groups, including 0 (NC), 40, 80 and 160 mg/kg ZnO NP groups, were obtained to evaluate the quadratic (Q) and linear (L) responses to levels of dietary ZnO NP. Growth performance was analysed using each pen as an experimental unit, while each
individual bird was used as an experimental unit for other parameters. \( p \) Values \(<.05\) indicated significant differences.

**Results**

In the current study, ZnO NPs showed nearly spherical geometry in low, intermediate and high magnification TEM images (Figure 1). The sizes of these ZnO NPs were mainly in 20 nm to 40 nm range.

Compared with the NC group, dietary 40–160 mg/kg ZnO NP increased the final body weight at 42 days \((p < .05, \text{Table 3})\). Supplementation with 40–160 mg/kg ZnO NP significantly improved ADG \((p\text{-linear } < .05; p\text{-quadratic } < .05)\) and feed-to-gain ratio \((p\text{-quadratic } < .05)\) in a dose-dependent manner during the 21–42 day and 1–42 day stages. In addition, dietary 160 mg/kg ZnO NP significantly increased ADFI and F:G compared to the ZnSO\(_4\) + Xanthomycin group during the 21–42 day and 1–42 day stages \((p < .05)\).

Supplementation with 40, 80, 160 mg/kg ZnO NP or 80 mg/kg ZnSO\(_4\) + 5 mg/kg xanthomycin did not significantly alter the organ index (thymus, spleen, bursa of fabricius and liver) compared to the NC group \((p > .05, \text{Table 4})\).

Supplementation with 40 mg/kg ZnO NP or 80 mg/kg ZnSO\(_4\) + 5 mg/kg xanthomycin significantly decreased the serum PC content compared to the NC group \((p < .05, \text{Table 5})\). Supplementation with ZnO NP dose-dependently decreased the serum PC content \((p\text{-linear } = .02; p\text{-quadratic } < .01)\). Compared with the NC group, the serum MDA level was significantly decreased by supplementation with 80 mg/kg ZnO NP or 80 mg/kg ZnSO\(_4\) + 5 mg/kg xanthomycin \((p < .05)\). Supplementation with ZnO NP also dose-dependently enhanced serum POD activity \((p\text{-linear } < .05; p\text{-quadratic } < .01)\), and supplementation with 40 or 80 mg/kg ZnO NP significantly increased serum POD activity compared to the NC group \((p < .05)\). However, no differences were noted in these serum antioxidant capacity parameters between the 80 mg/kg ZnO NP and ZnSO\(_4\) + Xanthomycin groups \((p > .05)\). As shown in Figure 2, compared with the NC group,

| Gene1  | Accession No. | Sequence (5’ to 3’) | Size (bp) |
|--------|---------------|---------------------|-----------|
| NQO1   | NM_001277621.1| F: GGCAATGGCAGCAGCAG | 138       |
|        | R: TGCATCTTGTCCACGCT       |                     |
| HO-1   | NM_205344.1   | F: ACAGTTCAAGTGCAGCAG | 244       |
|        | R: GTGACTTCTGGCAACACG       |                     |
| SOD    | NM_205064.1   | F: CGCGGTCGTAGGGGAGAT | 125       |
|        | R: TGCATCTTGTCCACGCT       |                     |
| CAT    | NM_001031215.2| F: GGTGCTGGGTGGTCCCTTT | 213       |
|        | R: CACCGTGTGTCAGCGGAT       |                     |
| GSH-Px | NM_001277853.2| F: GACCAACCCGAGTAGGATCA | 204       |
|        | R: ACTGTGCCGCGTCTTGTGCAGGC |                     |
| Nrf2   | NM_205117.1   | F: AGTTGGCTGAGAAGAGGGTG | 170       |
|        | R: AGTGGCTGAGAAGAGGGTG       |                     |
| Keap1  | KU321503.1    | F: AGCGGCGAGAGGTGGTATGA | 110       |
|        | R: GGATGCTTCTGGCAACACG       |                     |
| Occludin| NM_205128.1  | F: CGGTTAACCCCGAGTAGGAT | 214       |
|        | R: ATGAGGCCGCGTCTTGTGAG       |                     |
| Claudin-2| NM_001277622.1| F: CCTGCTCACCCTCAATTGAG | 145       |
|        | R: GCTGAAACTACCTTGGGCT       |                     |
| ZO-1   | XM_413773.4   | F: TGTAGCAAGCGAGTAGGCT | 159       |
|        | R: CTGGAAATGGCTCCTTGCTG       |                     |
| Mucin-2| XM_001234581.3| F: AGGATGGGCTCGGAAGAGAC | 77        |
|        | R: ATGCCCTACAGTTGGCTCGAGGC |                     |
| TLR4   | NM_001030969.1| F: CCTACACTACCCACACACAGA | 96        |
|        | R: TACACACCTGACTGTGGGAGGCG |                     |
| IFN-γ  | NM_205149.1   | F: CCTGACACCGGCGACACAGA | 87        |
|        | R: ATGAGCTGGCTGACACACACAGA |                     |
| IL-1β  | NM_204524.1   | F: GTACCGAGTACAACCCCTGCG | 112       |
|        | R: AGCAGGACGGAGCTAAGTAAAAG |                     |
| MYD88  | NM_001030962.1| F: ATCGCCGACACCATAGGGAGGAG | 115       |
|        | R: GGCAGAAGCTAGTGGCTCAGTTT |                     |
| TAK1   | NM_001006240.2| F: ACTGGGGTAAAGGAGTCCAC | 210       |
|        | R: ACCGGGTTAAAAGGAGTCCAC       |                     |
| IL-2   | XM_015276098.2| F: TTCTGGTCTGCGCTGTCCTGGG | 85        |
|        | R: TTCATGCACTGCCGAGGAGGAGGAGGAC |                     |
| AMPK   | CK610465.1    | F: GCACAGGAGAGGCAGTGCTT | 243       |
|        | R: TGCATCTTGGGGAGATGGTCCAC       |                     |
| β-actin| NM_205518.1   | F: TGGCTGGTCTCCTCAATCGCT | 150       |
|        | R: TTGGTACAACTAGCAGGGCTTGCTC       |                     

\(^{1}\text{AMPK, adenosine monophosphate-activated protein kinase; CAT, catalase; GSH-Px, glutathione peroxidase; HO-1, haem oxygenase-1; IFN-γ, interferon-γ; IL-2, interleukin-2; IL-1β, interleukin-1β; Keap1, kelch-like ECH associated protein 1; MYD88, myeloid differentiation primary response protein 88; NQO1, NAD(P)H dehydrogenase, quinone 1; Nrf2, nuclear factor erythroid-2-related factor 2; SOD, superoxide dismutase; TAK1, transforming growth factor-beta-activated kinase-1; TLR4, toll-like receptor 4; ZO-1, zonula occludens-1.}^{2}\)

**Figure 1.** Transmission electron microscopy images of ZnO NP at low (a), intermediate (b) and high (c) magnifications. ZnO NP, zinc oxide nanoparticle.
supplementation with 40–160 mg/kg ZnO NP caused a linear enhancement of mRNA expressions of HO-1, SOD, Nrf2, CAT and NQO1 (p-linear < .05), while supplementation with 80 or 160 mg/kg ZnO NP or the combination of 80 mg/kg ZnSO4 and 5 mg/kg xanthomycin significantly decreased Keap1 mRNA expression (p < .05). The mRNA abundances of these selected antioxidant-related genes did not significantly differ in the 3 ZnO NP and the ZnSO4 + Xanthomycin groups (p > .05).

As shown in Table 6, serum concentrations of GH, IGF-1, IL-10, IgA, IgG and IgM increased linearly (p-linear < .05) with the increasing level of dietary ZnO NP. Compared with the ZnSO4 group, the serum concentrations of GH, IGF-1, IgA, IgG and IgM increased linearly (p-linear < .05) with the increasing level of dietary ZnO NP.

Table 3. Effects of different treatments on broiler growth performance.

| Item | ZnSO4 + Xanthomycin | 0 (NC) | 40 | 80 | 160 | SEM^3 | p Value^a |
|------|---------------------|--------|----|----|-----|-------|-----------|
| BW 1 d (g) | 38.09 | 37.84 | 37.98 | 38.05 | 38.47 | 0.117 | .548 .135 .476 |
| 21 d (g) | 867.92 | 845.42 | 869.49 | 893.48 | 860.47 | 8.053 | .457 .257 .183 |
| 42 d (kg) | 2.69^bc | 2.60^bc | 2.80^a | 2.79^b | 2.76^a | 0.020 | .003 .003 .017 |
| ADG (g) | 1–21 d (g) | 39.52 | 38.45 | 39.60 | 40.74 | 39.14 | 0.381 | .451 .264 .176 |
| 21–42 d (g) | 86.67^bc | 83.78^bc | 91.99^a | 90.47^b | 90.68^a | 0.819 | .004 .003 .033 |
| 1–42 d (g) | 63.09^bc | 61.12^bc | 65.79^a | 65.60^b | 64.91^a | 0.473 | .003 .003 .016 |
| ADFI | 1–21 d | 56.26 | 53.57 | 54.42 | 57.74 | 56.15 | 0.558 | .130 .031 .692 |
| 21–42 d | 150.40^bc | 157.91^bc | 160.96 | 162.07^b | 175.93^a | 2.349 | .008 .009 .103 |
| 1–42 d | 103.33^bc | 105.74 | 107.69 | 109.91^b | 116.04^a | 1.231 | .008 .002 .138 |
| F: G | 1–21 d | 1.43 | 1.40 | 1.38 | 1.42 | 1.44 | 0.016 | .759 .330 .385 |
| 21–42 d | 1.74^bc | 1.88^bc | 1.75^a | 1.79^ab | 1.94^a | 0.022 | .007 .456 <.001 |
| 1–42 d | 1.64^bc | 1.73^bc | 1.64^a | 1.68^ab | 1.79^a | 0.017 | .018 .217 <.001 |

Table 4. Effects of different treatments on the organ indexes of broilers.

| Item | ZnSO4 + Xanthomycin | 0 (NC) | 40 | 80 | 160 | SEM^3 | p Value^a |
|------|---------------------|--------|----|----|-----|-------|-----------|
| Thymus (g/100 g BW) | 0.22 | 0.22 | 0.23 | 0.20 | 0.20 | 0.004 | .070 .017 .145 |
| Spleen (g/100 g BW) | 0.11 | 0.12 | 0.13 | 0.12 | 0.13 | 0.004 | .229 .247 .991 |
| Bursa of Fabricius (g/100 g BW) | 0.18 | 0.18 | 0.22 | 0.19 | 0.22 | 0.006 | .046 .103 .665 |
| Liver (g/100 g BW) | 1.92 | 1.81 | 1.94 | 1.87 | 1.94 | 0.030 | .646 .231 .715 |

Table 5. The effects of different treatments on the serum parameters of the antioxidant capacities of broilers.

| Item | ZnSO4 + Xanthomycin | 0 (NC) | 40 | 80 | 160 | SEM^3 | p Value^a |
|------|---------------------|--------|----|----|-----|-------|-----------|
| PC (nmol/mgprot) | 49.13^bc | 79.61^a | 33.56^c | 59.68^bc | 57.18^ab | 3.365 | <.001 .015 <.001 |
| MDA (nmol/mL) | 2.71^c | 4.32^a | 4.79^b | 2.86^bc | 4.14^b | 0.299 | <.001 .136 .846 |
| SOD (U/mL) | 323.75 | 294.71 | 316.04 | 321.08 | 331.29 | 4.410 | .089 .010 .869 |
| POD (U/mL) | 4.75^ab | 3.34^b | 5.02^a | 5.58^b | 4.23^a | 0.193 | <.001 .015 .001 |
| T-AOC (U/mL) | 7.64 | 7.16 | 7.22 | 7.04 | 6.89 | 0.198 | .820 .647 .722 |

Table 6. Serum concentrations of GH, IGF-1, IL-10, IgA, IgG and IgM.

| Item | ZnSO4 + Xanthomycin | 0 (NC) | 40 | 80 | 160 | SEM^3 | p Value^a |
|------|---------------------|--------|----|----|-----|-------|-----------|
| PC (nmol/mgprot) | 49.13^bc | 79.61^a | 33.56^c | 59.68^bc | 57.18^ab | 3.365 | <.001 .015 <.001 |
| MDA (nmol/mL) | 2.71^c | 4.32^a | 4.79^b | 2.86^bc | 4.14^b | 0.299 | <.001 .136 .846 |
| SOD (U/mL) | 323.75 | 294.71 | 316.04 | 321.08 | 331.29 | 4.410 | .089 .010 .869 |
| POD (U/mL) | 4.75^ab | 3.34^b | 5.02^a | 5.58^b | 4.23^a | 0.193 | <.001 .015 .001 |
| T-AOC (U/mL) | 7.64 | 7.16 | 7.22 | 7.04 | 6.89 | 0.198 | .820 .647 .722 |
broilers from 40, 80 and 160 mg/kg ZnO NP groups were lower \((p < .05)\), while serum contents of GH, IgA, IgG and TNF-\(\alpha\) showed no differences between the 160 mg/kg ZnO NP and ZnSO\(_4\) + Xanthomycin groups \((p > .05)\).

As shown in Figure 3, supplementation with 40–160 mg/kg ZnO NP did not significantly influence mRNA expressions of IL-1\(\beta\), TAK1 or MYD88 \((p > .05)\), while 40 or 80 mg/kg ZnO NP significantly decreased the mRNA abundance of IFN-\(\gamma\), TLR4 and IL-2 \((p < .05)\),
and 160 mg/kg ZnO NP significantly enhanced AMPK mRNA expression (p < .05). No significant differences were noted in the mRNA abundance of these genes among the ZnSO4 + Xanthomycin group and the 3 ZnO NP groups (p > .05).

As shown in Table 7, supplementation with 40, 80 or 160 mg/kg ZnO NP significantly decreased serum DAO activity and D-lactate content (p < .05). The endotoxin content was significantly decreased by supplementation with 40 mg/kg ZnO NP (p < .05). Serum D-lactate content (p-linear < .05; p-quadratic < .05), DAO activity (p-linear < .05; p-quadratic < .05) and endotoxin content (p-linear < .05) dose-dependently decreased with the increasing level of dietary ZnO NP. No differences in serum endotoxin content or DAO activity were found among the ZnSO4 + Xanthomycin group and the 3 ZnO NP groups (p > .05).

As shown in Figure 4, ZO-1 and claudin2 mRNA expressions were higher in 160 mg/kg ZnO NP group than that in the NC group (p < .05). The mRNA expressions of ZO-1 (p-linear < .01), claudin-2 (p-linear
and mucin 2 (\(p\)-linear < .01) dose-dependently enhanced with the increasing level of dietary ZnO NP. No differences were noted in the mRNA expressions of these selected genes in the ZnSO4 + Xanthomycin group and the 3 ZnO NP groups (\(p > .05\)). Furthermore, the jejunal villus height (\(p\)-linear < .05) and villus width (\(p\)-linear < .05) dose-dependently increased with the increasing level of dietary supplementation with ZnO NP (Table 8 and Figure 5). Compared with the NC group, supplementation with 80 mg/kg ZnO NP increased the villus width and height (\(p < .05\)). The jejunal morphology showed no significant differences among 80, 160 mg/kg ZnO NP and ZnSO4 + Xanthomycin groups (\(p > .05\)). As shown in Table 8 and Figure 6, supplementation with 40–160 mg/kg ZnO NP or 80 mg/kg ZnSO4 + 5 mg/kg xanthomycin significantly enhanced the number of jejunal goblet cells compared with the NC group (\(p < .05\)).

As shown in Table 9, no differences were detected in the serum activities of GOT, GPT and AKP among the 5 groups (\(p > .05\)). Hepatic histomorphology analysis (Figure 7) also showed no significant changes in hepatic morphology among the 5 groups (\(p > .05\)).

**Figure 4.** Effects of different treatments on mRNA expressions of occludin, ZO-1, claudin-2, and mucin2 in the jejunal mucosa of broilers. NC, negative control; ZnO NP, zinc oxide nanoparticle; ZO-1, zonula occludens-1. ZnSO4 + Xanthomycin group, basal diet supplemented with 80 mg/kg ZnSO4 and 5 mg/kg xanthomycin; NC group, basal diet; 40, 80 and 160 mg/kg ZnO NP group, basal diet supplemented with 40, 80 and 160 mg/kg ZnO NP, respectively. The column and its bar represented the means value and SEM (\(n = 8\) birds). a, b within the same gene, means with different superscripts differ (\(p < .05\)). Q and L are the quadratic and linear responses, respectively, to levels of dietary supplementation with ZnO NP.

**Table 8.** Effects of different treatments on jejunal morphology and goblet cell number in broilers.

| Item 1 | ZnSO4 + Xanthomycin 2 | ZnO NP (mg/kg) | \(p\) Value 2  |
|--------|-----------------------|----------------|----------------|
| Villus height (\(\mu m\)) | 1487.38⁰ | 1282b | 1263.58b | 1503.34a | 1391.78ab | 28.466 | .002 | .011 | .953 |
| Villus width (\(\mu m\)) | 178.19ab | 174.82b | 177.63ab | 191.16a | 181.88ab | 1.809 | .021 | .039 | .392 |
| Crypt depth (\(\mu m\)) | 248.29 | 247.72 | 244.76 | 243.02 | 240.09 | 5.091 | .990 | .699 | .950 |
| Villus height/crypt depth (\(\mu m/\mu m\)) | 6.01 | 5.18 | 5.24 | 6.22 | 5.86 | 0.155 | .101 | .053 | .980 |
| the number of goblet cells | 185.51a | 138.89c | 175.89ab | 173.41ab | 162.22b | 4.089 | <.001 | <.001 | <.001 |

1NC, negative control; ZnO NP, zinc oxide nanoparticle.
2ZnSO4 + Xanthomycin group, basal diet supplemented with 80 mg/kg ZnSO4 and 5 mg/kg xanthomycin; NC group, basal diet; 40, 80 and 160 mg/kg ZnO NP group, basal diet supplemented with 40, 80 and 160 mg/kg ZnO NP, respectively.
3SEM, pooled standard error of the means.
4°Q and L are the quadratic and linear responses, respectively, to levels of dietary supplementation with ZnO NP (\(n = 4\) birds).
5Means in a row with different superscripts are significantly different as \(p < .05\).
Figure 5. Effects of different treatments on the jejunal morphology of broilers (haematoxylin and eosin staining images). NC, negative control; ZnO NP, zinc oxide nanoparticle. ZnSO₄ + Xanthomycin group, basal diet supplemented with 80 mg/kg ZnSO₄ and 5 mg/kg xanthomycin; NC group, basal diet; 40, 80 and 160 mg/kg ZnO NP group, basal diet supplemented with 40, 80 and 160 mg/kg ZnO NP, respectively. The length of the red line segments indicates the villus height (from tip of villus to the crypt opening, μm), the length of the yellow line segments indicates the crypt depth (from the base of the crypt to the level of crypt opening, μm), the average value of the length of the green line segments indicates the villus width (at half of height, μm).

Figure 6. Effects of different treatments on jejunal goblet cells of broilers (periodic acid-Schiff staining images) of broilers. NC, negative control; ZnO NP, zinc oxide nanoparticle. ZnSO₄ + Xanthomycin group, basal diet supplemented with 80 mg/kg ZnSO₄ and 5 mg/kg xanthomycin; NC group, basal diet; 40, 80 and 160 mg/kg ZnO NP group, basal diet supplemented with 40, 80 and 160 mg/kg ZnO NP, respectively. Arrows indicate purplish red goblet cells.
Discussion

ZnO NP exhibits multiple properties, such as antibacterial activity, which are closely associated with their characteristics and have been widely reviewed (Gajjar et al. 2009; Sirelkhatim et al. 2015). The TEM images indicated that the ZnO NP used in this study was nearly spherical nanoparticles with sizes ranging from 20 to 40 nm, in agreement with the ZnO NP used in our previous work on mice and piglets (Wang et al. 2016; Wang et al. 2018a).

Our present results suggested that dietary 40–160 mg/kg ZnO NP showed comparable effects on the growth performance of broilers to those achieved with the combination of 80 mg/kg ZnSO₄ and 5 mg/kg xanthomycin. These findings confirmed that dietary supplementation with ZnO NP, as a novel multiple nano-material, may substitute the combination of antibiotics and ZnSO₄ (Wang et al. 2017b; Sizova et al. 2020). Compared with the NC group, 40–160 mg/kg ZnO NP significantly increased the final body weight (42 days of age) and ADG during the 21–42 day and 1–42 day stages. However, the F:G ratio was higher for broilers supplemented with 160 mg/kg ZnO NP than with 40 mg/kg ZnO NP, indicating that doses of 40–80 mg/kg ZnO NP might be the optimal level for broiler diets. This finding agreed with the results from Zhao et al. (2014), who reported that 20–60 mg/kg ZnO NP could improve body weight gain and feed conversion ratio in broiler chicks compared to 50 mg/kg traditional ZnO. Sizova et al. (2020) also proposed that a nanoscale form of zinc could replace the organic and inorganic forms in poultry diets. Except for the high absorption, ZnO NP also shows multiple properties, such as wound healing, antimicrobial and anti-inflammatory activity, and regulating hormones (Baltaci et al. 2019; Agarwal and Shanmugam 2020;
Sizova et al. 2020). The GH and IGF-1 hormones have important biological functions, especially in growth performance, through regulation of nutrient metabolism (Scanes 2009; Brooks et al. 2014). In the current study, the serum GH and IGF-1 concentrations were increased linearly with the increasing levels of dietary ZnO NP. Dietary 40–160 mg/kg ZnO NP significantly increased the serum IGF-1 level as compared with the NC group, which was in agreement with the results from Swain et al. (2021), who reported that dietary nano zinc improved the serum IGF-1 hormones in goat. GH can induce the production of IGF-1, and the latter further promotes protein synthesis and improves animal growth or development (Dishon et al. 2021). Taken together, our results suggested that the beneficial effects of ZnO NP on the growth performance of broilers may, at least in part, contribute to the increased serum levels of GH and IGF-1 (Baltaci et al. 2019; Dishon et al. 2021).

In the current study, supplementation with 40–160 mg/kg ZnO NP did not significantly change the indices of selected organs, including the thymus, spleen, bursa of fabricius and liver, compared to the NC group. The same trend has been reported in mice, where dietary supplementation with 50–500 mg/kg ZnO NP did not alter the relative organ weights (Wang et al. 2016). These organ indices are related to the capacity to produce immune cells, but they are also critical indicators for evaluating the negative effects of drugs or additives (Bailey et al. 2004; Kidd 2004). Our results suggested that supplementation with 40–160 mg/kg ZnO NP did not show obvious negative effects on the organ development in broilers, in agreement with previous reports on weaned piglets (Wang et al. 2017a; Wang et al. 2018a).

The MDA and PC contents are important indicators of lipid peroxidation and protein oxidation and commonly show negative correlations with the antioxidant capacity in animals (Hawkins and Davies 2019). In our current study, supplementation with 40 or 80 mg/kg ZnO NP decreased the serum MDA and PC contents compared with the NC group, indicating that supplemental ZnO NP could promote the serum antioxidant capacity of broilers. We also found that supplementation with ZnO NP dose-dependently increased the serum POD activity, suggesting that the beneficial effects of supplemental ZnO NP on the antioxidant capacity may be related to the enzyme antioxidant system. This possibility is consistent with the results from Zhao et al. (2014), who verified that ZnO NP at appropriate levels could improve the antioxidant capacity and antioxidant enzyme activities of broilers, whereas excessive level of ZnO NP did not promote serum antioxidant activity. In cells, antioxidant activity is commonly controlled by the Nrf2/HO-1 signal pathway (Reziwan et al. 2019). Nrf2 and its negative regulator Keap1 are the central regulators of cellular antioxidant responses (Kobayashi et al. 2004). The Nrf2 escaping Keap1-mediated repression can induce the production of some antioxidant enzymes, including SOD, CAT, HO-1, by increasing the corresponding expression levels (Kobayashi et al. 2004; Zhang 2006; Jiang et al. 2017). Our findings indicated that supplementation with ZnO NP enhanced the mRNA expressions of several members of Nrf2/HO-1 signal pathway, including HO-1, SOD, Nrf2, CAT and NQO1, together with a dose-dependent down-regulation of Keap1 expression. The Nrf2/HO-1 pathway is also involved in anti-inflammation and is regulated by genes, antioxidants or nutrients (Kosuru et al. 2018; Reziwan et al. 2019). Agents activating Nrf2 pathway have been shown to down-regulate the overproduction of pro-inflammatory cytokines such as IL-2, TNF-α, and IFN-γ, and up-regulate the production of immunoglobulins including IgA, IgG and IgM (Kim et al. 2010; Wang et al. 2018b). Among the enzymes up-regulated by Nrf2, HO-1 is an important cytoprotective enzyme, modulating anti-inflammatory and antioxidative processes (Kim et al. 2010). TLR4 and AMPK are also closely associated with the inflammatory response (Tadie et al. 2012). Our results showed that the mRNA expressions of IFN-γ, TLR4 and IL-2 in the jejunal mucosa and serum TNF-α concentration were decreased, and AMPK mRNA expression in the jejunal mucosa and serum IL-10 content were increased as the supplementation level of ZnO NP was increased. These results are consistent with previous reports that up-regulated Nrf2/HO-1 signalling can inhibit TLR4-mediated inflammatory responses (Rao et al. 2015; Liu et al. 2019). In addition, the serum contents of IgA, IgG and IgM showed significant dose-dependent increases in response to supplementation with ZnO NP, suggesting an enhancement of non-specific immunity that could aid in alleviating environmental stress and encouraging rapid broiler growth by neutralising pathogens. A previous study showed that the non-specific defence system can be inhibited by oxidative stress (Ercal et al. 2000). Therefore, the improvement of the antioxidant status, the enhancement of non-specific immunity and the inhibition of inflammation in broilers by ZnO NP may be involved in the Nrf2/HO-1 signal pathway, in agreement with the results from Barakat et al. (2020).
The intestinal barrier is a critical barrier against pathogens in the lumen and delivers nutrients safely to the circulatory system (Nakajima et al. 2020). In this study, the ZnO NP dose-dependently decreased the contents of D-lactate and endotoxin, and reduced DAO activity in serum compared with the NC group, suggesting that supplementation with ZnO NP protected the intestinal barrier of broilers (Zhou et al. 2004; Tossou et al. 2016). We also found a dose-dependent enhancement of mRNA expressions of ZO-1 and claudin2 in the jejunal mucosa by supplementation with ZnO NP compared with the NC group. Claudin2 and ZO-1 are important components of tight junction proteins, and their loss can reduce the integrity and increase the permeability of the intestine (Turner 2009). The present findings indicated that the protective effects of ZnO NP on the mucosal barrier might be attributable to the enhanced mRNA expression of genes related to tight junctions (Wang et al. 2017b; Wang et al. 2018a). The jejunal villus height and villus width increased with increasing levels of dietary ZnO NP, indicating that dietary ZnO NP improved the jejunal morphology, and may further improve nutrient absorption and enhance the integrity of the mucosal barrier (Shao et al. 2013). Goblet cells can synthesise and release mucins to form a mucus layer that covers and protects intestinal epithelial cells (Johansson et al. 2013). Results of the current study indicated that 40–160 mg/kg ZnO NP significantly increased the number of jejunal goblet cells compared with the NC group. Taken together, our results suggested that supplementation with ZnO NP could improve the intestinal barrier, morphology and mucus layer to protect the gut health of broilers, and this might explain the observed beneficial effects on growth performance (Ercal et al. 2000).

ZnO NP has multiple beneficial properties and low cost; however, their potential damages to animals or humans, especially in the liver, might be the greatest limitation hampering the use of ZnO NP as feed additives (Sabir et al. 2014; Gharpure and Ankamwar 2020). The serum activities of GOT, GPT and AKP were determined to evaluate the liver function. We found no alterations in the serum activities of these enzymes following ZnO NP supplementation, indicating that supplementation of ZnO NP at levels of 40–160 mg/kg for 42 days did not cause obvious negative effects on liver function in broilers. The same trend was evident in the hepatic histomorphology study, which showed no significant changes in hepatic morphology of broilers among the 5 groups. These results are in line with our previous studies in mice and piglets (Wang et al. 2016; Wang et al. 2017a; Wang et al. 2018a). Mahmoud et al. (2021) also reported that long-term supplementation with low-dose (<40 mg/kg diet) ZnO NP did not show observed adverse effects on liver histology, blood physiology, immune system, and DNA damage of liver in broilers.

**Conclusion**

Dietary supplementation with 40–160 mg/kg ZnO NP caused no obvious negative effects on liver function of broiler chickens, but effectively improved the growth performance and enhanced immunity, antioxidant capacity and intestinal barrier function in broilers. The beneficial effects of dietary ZnO NP were comparable to those of the combination of 80 mg/kg ZnSO4 and 5 mg/kg xanthomycin in broiler diet. Considering the feed costs and beneficial effects, ZnO NP doses at 40–80 mg/kg are recommended in broiler diets and ZnO NP, especially at levels of 80 mg/kg, could substitute the combination of 80 mg/kg ZnSO4 and 5 mg/kg xanthomycin, thereby facilitating the elimination of antibiotics and utilisation of ZnO NP in broiler feeds.

**Ethical approval**

All the experimental procedures and animal care protocols were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) and was conducted under the corresponding supervision (Permit number: SYXK-2019-00085).

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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