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Impact of Oral Administration of Chitosan–nanoparticles on Oxidative Stress Index and Gut Microbiota of Heat Stressed Broilers

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Abstract

The study investigated the effect of the administration of chitosan–nanoparticles (ChNP) on body performance, gut microbiota, some immunological parameters, expression of digestibility, and antioxidant-related genes in broilers. A total of 80 one-day-old Ross mixed-sex chicks were divided into two groups (40 birds each) in open door system: Chicks in group 1 were fed a basal diet as control, whereas chicks in group 2, were fed a basal diet, and drinking water supplemented with 50-mg ChNP/L. The experimental period lasted three weeks with a daily observation of body performance. Serum and tissue samples were collected for immunological evaluation, microbiota counts, and estimation of mRNA expression levels. ChNPs supplementation significantly increased beneficial microbiota and diminished coliforms, improved growth performance, kidney functions, total antioxidant capacity, and gene expressions of *Glut, CAT1, CAT, SOD*, and *GPX1* in broilers. In conclusion, supplementation of ChNP led to an upregulation of body performance and antioxidant activity of broiler chicks phenotypically and genotypically.

KEYWORDS
Antioxidant, Broilers, Chitosan nanoparticles, Gene expression, Gut microbiota.

INTRODUCTION

Recently, there has been an unexpected rise in worldwide ambient temperature which has significant implications for the farming sector in both tropical and subtropical locations. As temperatures over the typical range (thermo-neutral zone) in living organisms disrupt normal biological processes and cause cell damage. High ambient temperatures frequently result in stress-related issues such as reduced production, metabolic abnormalities, growth retardation, and lack of effectiveness (Afsal *et al*., 2018; Nawaz *et al*., 2021).

In the poultry industry, heat stress is a significant issue that impacts the productivity and health of poultry. In 2003, heat stress caused economic losses ranging between $128 to $165 million (St-Pierre *et al*., 2003) and as the world's temperature rises, this number will increase annually.

High ambient temperature, high environmental temperature, humidity, radiant heat, and airspeed are numerous variables that collaborate to cause heat stress. In detail, the chicken's body temperature is often between 41 and 42°C whereas the ideal temperature for bird growth is between 18 and 21°C. Concerning several studies, birds suffer from heat stress at any ambient temperature more than 25°C (Wasti *et al*., 2020), and are unable to maintain their body's heat production and loss creating oxidative stress due to the production of reactive oxygen species (ROS) (Naga Raja Kumari and Narendra Nath, 2018).

In heat-stressed broilers, significant changes in the gut microbial load have been recorded, including lower abundances of beneficial bacteria (such as *Lactobacillus* spp.) and an increase in coliforms and *Clostridium* spp. (Song *et al*., 2014). From this point, heat stress might lead to dysbiosis and a rise in intestinal permeability, metabolic impairment, and lowered immune response (Zhu *et al*., 2019).

In studies conducted by Adhikari *et al*. (2020); Dawwam *et al*. (2022) and Sharaf *et al*. (2022), nanoparticles, which can be either natural or synthetic and range in size from 1 to 100 nanometers, have demonstrated their effectiveness as antioxidant agents and in the treatment of several avian diseases.

Chitosan is a beneficial polymer for numerous applications, including agriculture, food science, pharmaceuticals, and biomedical disciplines because of its distinctive combination of qualities, including biodegradability, renewable energy, and biofunctional activity (Yin *et al*., 2009). Chitosan contains reactive functional groups that have been shown to have antioxidant, anti-inflammatory, antibacterial, and immunostimulatory effects (Holappa *et al*., 2006; Zaharoff *et al*., 2007; Yen *et al*. 2008; Ma *et al*., 2011).

Chitosan can be used as a potent source of antioxidants for broiler chickens since it possesses antioxidant characteristics, according to Anraku *et al*. (2018). Applications of nano-chitosan, a natural material with exceptional physicochemical properties, have been created to improve the growth performance, immune status, and microflora of commercial poultry birds (Xu *et al*., 2018). Chitosan increases antioxidant activity in laying chicks'

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blood, liver, and duodenum to varying degrees. However, insufficient studies have examined the modulatory effect of chitosan on the expression of genes related to antioxidant activity and growth performance in broilers.

Hence, the present study was designed to validate the efficacy of nano-chitosan supplementation on the development of intestinal microbiota, some immune-parameters, body performance, and antioxidant activity phenotypically and genotypically in heat-stressed broilers.

MATERIALS AND METHODS

Ethical Approval

The care and bird used institutional guidelines followed according to the rules approved by the ethics of the Local Committee Institute (ARC-IACUC): Animal Health Research Institute Ethical Committee Approval Number: ARC/AH/21/10.

Nano-chitosan preparation

Chitosan nanoparticles (ChNP) were created using a modified version of Calvo *et al*. (1997) ionic gelation process. The method relies on the electrostatic interaction of an amine group in chitosan (Sigma-Aldrich, USA; molecular weight 50,000–190,000 Da; degree of deacetylation: 75–85%; viscosity: 20–300 cP) and a negatively charged compound, such as sodium tripolyphosphate (TPP) (Sigma-Aldrich, USA). DNA-free deionized water (Millipore, USA) was used for preparation and dilutions. Chitosan (Ch) was dissolved in acetic acid solution (1% v/v) at room temperature to produce Ch aqueous solution (0.2% w/v). The TPP solution (0.06% w/v) was then dropped into the Ch solution and vigorously stirred for a further 30 minutes. The resultant chitosan particle suspension underwent 30-minute centrifugation at 12000 g. In deionized water, the pellet was reconstituted.

Approximately 0.25 grams of low-molecular-weight chitosan powder, which is 93% deacetylated, were dissolved in 230 ml of distilled water, and 2.5 ml of acetic acid, and 20 minutes later the pH was adjusted to 4.6-4.8 using NaOH.

Twenty ml of an aqueous tripolyphosphate (TPP) was added to the chitosan beaker and stirred magnetically for 45 minutes to produce a white precipitate that served as a sign of the spontaneous formation of nano-chitosan.

Centrifugation was used to clean the nanoparticles for 30 minutes at 9000 rpm. Before using or analyzing the nano-chitosan for further research, the supernatants were discarded, and the material was thoroughly rinsed with distilled water to eliminate any sodium hydroxide.

Characterization of chitosan nanoparticle

The morphology (shape and size) of obtained nano-chitosan particles was examined using transmission electron microscopy (TEM). An amorphous carbon layer was placed after the colloidal solution drop was deposited on a copper grid. It was analyzed using a TEM without any stains after being allowed to dry at room temperature (Hu *et al*., 2002).

Experimental design

The experiment was carried out in an open-door flock in an open-door system between July and August 2022. A total of 80 one-day-old chicks (Ross 308) were divided randomly into two experimental groups, with four replicates of each treatment and ten chicks in each duplicate.

As a control (Group 1); birds were fed a base diet, whereas birds in Group 2 were fed a base diet and received their water supplemented with 50 mg ChNP/L (Abd El-Ghany *et al*., 2021).

The experiment was lasted for three weeks. The base diet did not contain any antibiotic growth promoters. Water and Feed were available *ad libitum*.

Sampling

Blood samples were collected from the wing vein of each bird in a clean, dry centrifuge tube without anticoagulant, left to clot at room temperature, and then centrifuged at 3000 rpm for 15 min. The sera were obtained for the evaluation of some biochemical and antioxidant parameters.

Euthanasia was conducted using a gaseous concentration of 45% carbon dioxide to gently blow out the chicks (Wang *et al*., 2021).

Intestine and liver samples from each chicken were subsequently collected for bacterial count examination and gene expression analysis under complete septic conditions.

Biochemical analysis

Analysis included estimation of total protein (TP), albumin (Alb), and measurement of globulins (Glob) by subtracting albumin values from STP. The values of creatinine (Creat), blood urea nitrogen, and also calcium, and phosphorus were determined using a semiautomatic biochemical analyzer and commercially available colorimetric test kits (Biodiagnostic CO, Egypt) calculated according to the manufactured structure.

Blood serum total antioxidant capacity (TAC) concentration was assessed using a standardized kit (Biodiagnostic, Dokki- Giza, Egypt) based on the interaction of the sample's antioxidants with a specific volume of exogenously supplied hydrogen peroxide (H_2O_2) (Koracevic *et al*., 2001).

Blood serum total peroxide (TPX) concentration was calculated as the blood serum H_2O_2 equivalent using the Erel (2005) approach.

Oxidative Stress Index (OSI) was determined using the following formula for the calculation: [(TOS, umol/L)/(TAC, mmol equivalent/L) x100] (Erel, 2005).

Microbial count

The intestinal contents were collected, deposited in sterile sample bags, and then transferred immediately to the lab to be analyzed bacteriologically in a cooler box with ice packs (-4 to -10°C). According to Reuben *et al*. (2019), the sample contents were serially diluted from 10^{-1} to 10^{-7} to determine the quantity of *Lactobacillus* and *E. coli* prevalent. Then,0.1 ml of each diluted sample was placed in the proper agar media and incubated under the appropriate circumstances. CFU/g unit was used to express enumeration data.

Reverse transcription-quantitative real-time PCR (RT-qPCR) approach for genes expression analysis

RNA was extracted from tissue samples using a QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH, Cat. No. 74904) for expression analysis by RT-qPCR assessment. The expression levels of β-actin (housekeeping gene) were normalized within samples using this assay. The total volume of the reaction was 20 µL including 10 µL of the 2x HERA SYBR® Green RT-qPCR Master

Mix (Willowfort, UK), 1 µL of RT Enzyme Mix (20X), 0.5 µL of each primer at a concentration of 20 pmol, 3 µL of water, and 5 µL of RNA template. Step one real-time PCR equipment was used to carry out the reaction in the Biotechnology Unit, Animal Health Research Institute, Zagazig Branch, Egypt under the conditions listed in Table 1 and the Ct value was used to calculate the variation in RNA expression levels of tested samples (Yuan *et al*., 2006).

Statistical Analysis

The Levene and Shapiro–Wilk tests were utilized to check for normality and homogeneity of variance (Razali and Wah, 2011). The differences between the two groups for gene expression, body performance and biochemical parameters measurements were detected by paired T-test (Stokes *et al*., 2012). Results were expressed as means±SE and statistical significance was accepted at a probability less than 0.05. GraphPad Prism software (version 8; GraphPad Software Inc.) was utilized to generate graphical outputs.

RESULTS

Particle size and morphology of nano-chitosan

Figure 1 shows the size, dispersion, and uniformity of nano chitosan with particle diameter around 100nm.

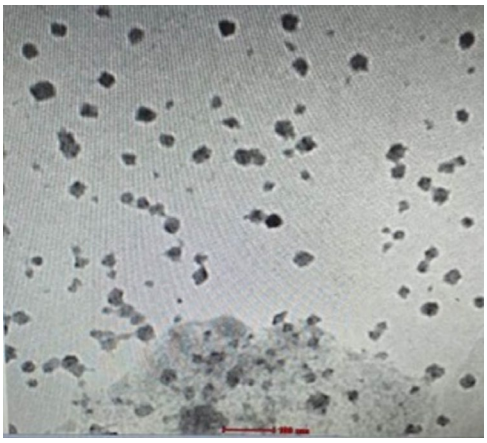

Fig. 1. TEM image showing the nearly spherical shape of prepared chitosan nanoparticles.

Clinical signs and mortality

Both groups behaved normally and exhibited no clinical signs or symptoms. No mortalities in either group were recorded during the entire research that lasted 0–21 days.

Microbial populations

Results in Fig. 2-A revealed that the coliform count was significantly decreased in the nano chitosan-treated group compared to the control group ($p<0.01$), whereas *Lactobacillus* count did not increase significantly by the treatment (Fig. 2B; $p>0.05$).

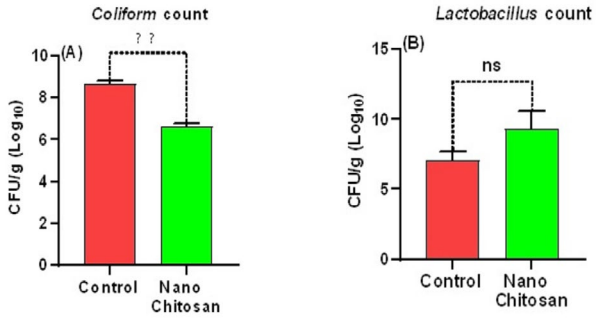

Fig. 2. Effect of administration of chitosan nanoparticles on bacterial count (Coliform, A; *Lactobacillus*, B) compared to the control group.

Growth performance

Both body and bursa weights and their ratio were improved significantly by the treatment ($p<0.01$; Fig. 3A-C).

Biochemical indices

Blood protein and globulin were significantly improved in the treated groups compared to the control, the significant differences were estimated as 0.6830 and 0.5990, respectively ($p<0.05$; Fig. 4A and C). Meanwhile the concentration of blood albumin did not influence significantly by the treatment ($p>0.05$; Fig. 4B). Concerning nutrition elements, the concentration of calcium did not increase significantly by nano chitosan treatment ($p>0.05$; Fig. 5A), while the phosphorus level was increased significantly ($p<0.05$; Fig. 5B). For kidney functions, there were no significant effects on the concentration of blood urea ($p>0.05$; Fig. 6-A) but the concentration of creatinine was significantly higher in the treated group than in the control ($p<0.05$; Fig. 6B). Oxidant and antioxidant parameters were improved signifcantly by the treatment, both TP and OSI were significantly diminished in the treated group compared to the control ($p<0.05$; Fig. 7A and C). In contrast; total antioxidant capacity was significantly higher in the treated group than in the control ($p<0.01$; Fig. 7B).

Table 1. Target genes, primers sequences, and cycling conditions for SYBR green rt-PCR.

Target gene	Primers sequences	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Reference
				Secondary denaturation	Annealing (Optics on)	Extension	
β-actin	CAACACAGTGCTGTCTGGTGG ATCGTACTCCTGCTTGCTGAT	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	60˚C 30 sec.	72˚C 30 sec.	Abdul-Careem *et al*. (2008)
Glut	TCCTCCTGATCAACCGCAAT TGTGCCCCGGAGCTTCT						Abdul-Careem *et al*. (2008)
CAT1	CAAGAGGAAAACTCCAGTAATTGCA AAGTCGAAGAGGAAGGCCATAA						Abdul-Careem *et al*. (2008)
SOD1	CACTGCATCATTGGCCGTACCA GCTTGCACACGGAAGAGCAAGT						Ahmadipour and Khajali (2019)
CAT	TGGCGGTAGGAGTCTGGTCT GTCCCGTCCGTCAGCCATTT						Ahmadipour and Khajali (2019)
GPX1	GCTGTTCGCCTTCCTGAGAG GTTCCAGGAGACGTCGTTGC						Ahmadipour and Khajali (2019)

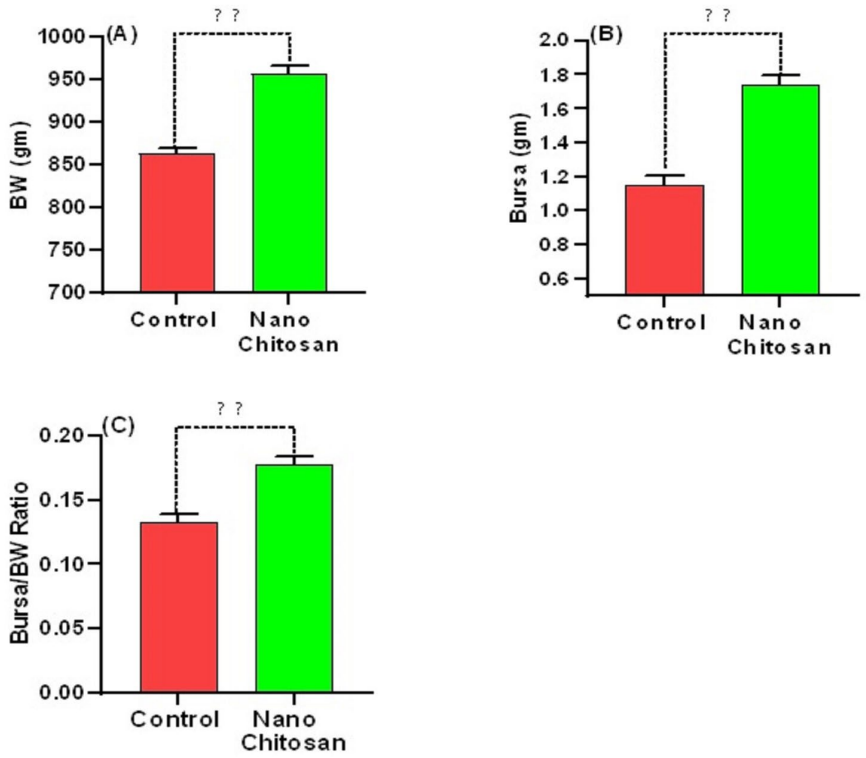

Fig. 3. Effect of administration of chitosan nanoparticles on body performance (BW, A; Bursa, B; Bursa/BW, C) compared to the control group.

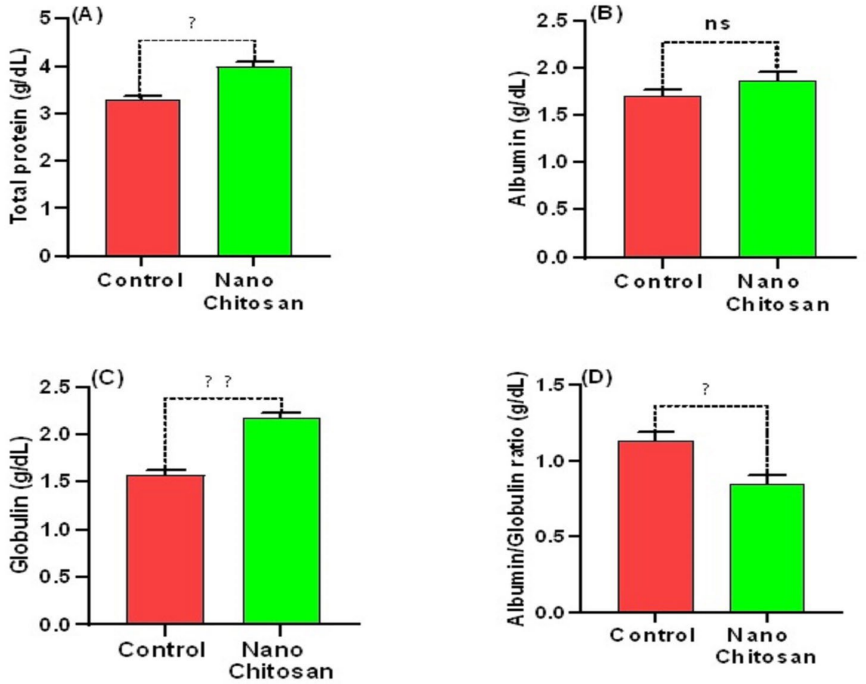

Fig. 4. Effect of administration of chitosan nanoparticles on blood protein (Total protein, A; Albumin, B; Globulin, C; Albumin/Globulin ratio, D) compared to the control group.

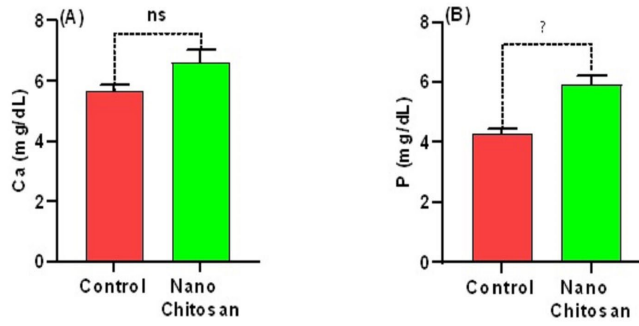

Fig. 5. Effect of administration of chitosan nanoparticles on nutrition elements (Calcium, A; Phosphorus, B) compared to the control group.

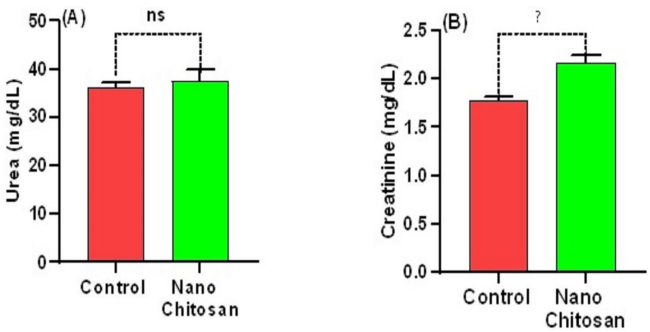

Fig. 6. Effect of administration of chitosan nanoparticles on kidney function (Urea, A; Creatinine, B) compared to the control group.

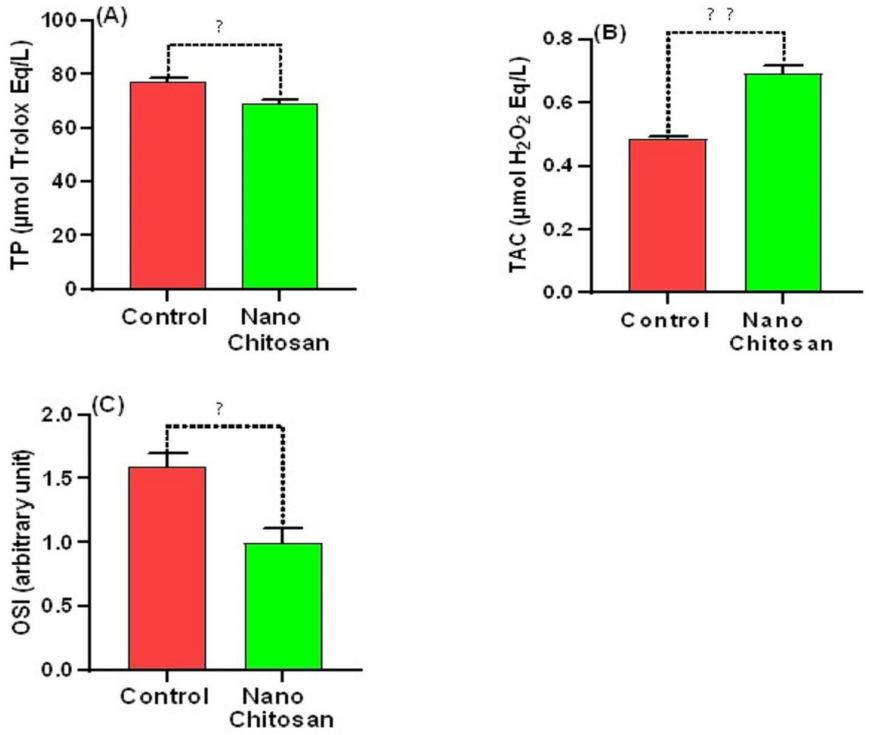

Fig. 7. Oxidant and antioxidant parameters in administrated ChNP and control groups.

Transcriptional modulatory effect of Nano–Chitosan administration

Nano–Chitosan treatment excreted significant effects on the transcription of all studied genes with differences of 3.551, 1.461, 2.635, 2.911, and 3.351, for Glut, CAT1, SOD1, CAT, and GPX1, respectively compared to the control group (Fig. 8).

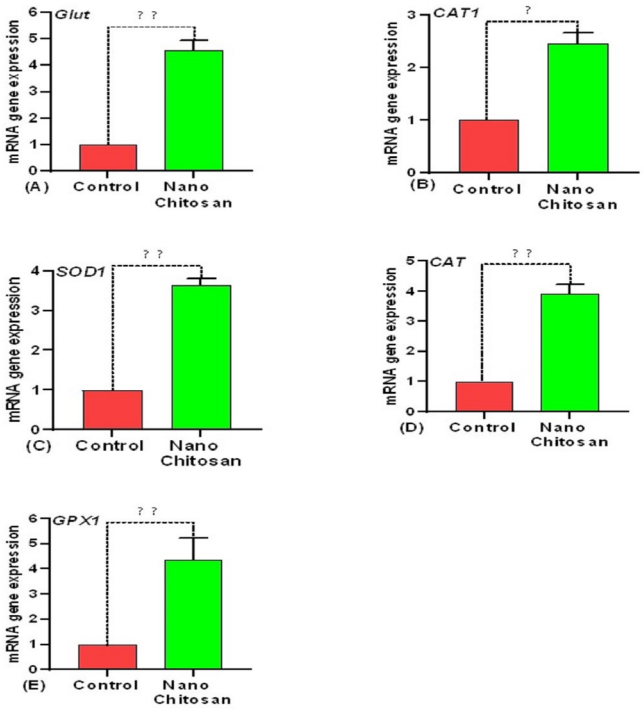

Fig. 8. The relative gene expression levels of the growth performance and antioxidant activity genes to the β-actin gene through administrated ChNP group in comparison to the control group.

DISCUSSION

To date, the relation between the caecal microbiota, and predisposing factors including heat stress have not been studied enough concerning the etiology of infections and response to any treatment.

It is widely acknowledged that the gut microbiota is essential for the host animals' physiological and dietary requirements (Rehman *et al.*, 2008). The lactobacilli in broiler intestines are beneficial to the hosts, whereas coliforms are detrimental (Santos *et al.*, 2006).

From this point, heat stress might lead to dysbiosis and increased intestinal permeability, which leads to disturbance in microbial load, metabolic impairment, and lower immune response (Song *et al.*, 2014; Wasti *et al.*, 2020; Nawaz *et al.*, 2021).

Microbiological examination on day 21 revealed that ChNP supplementation to the drinking water increased a load of lactobacilli in the chickens' cecum and decreased the count of coliforms. Tamara *et al.* (2018) and Xu *et al.* (2020) ascribed these findings to the ability of ChNP to induce the formation of biofilm-like structures in coliforms and improve the colonization of the GIT by lactobacilli. Thus, ChNP will promote health, productivity, and performance as this help in the digestion of dietary proteins, carbohydrates, and fats.

As follow, a significant increase in the body weights (BW) and weight of the bursa of broiler chickens were recorded supporting the previously documented mechanisms. The ratios of bursa weights to BW are very important indexes for the immune response of organs (Li *et al.*, 2015). In this study, the bursa of the Fabricius index was significantly increased by adding ChNP revealing a powerful immune rejoinder as mentioned by (Scott *et al.*, 2018).

It's noteworthy that ChNP markedly raised the TP, ALB, Glob, and A/G ratio serum protein synthesis-related indices. This improvement in protein synthesis may be the main factor for enhanced growth performance and decreased urea content in serum (Wang *et al.*, 2011). These results explain the notable increase in the weight of the bursa and its ratio with body weight.

In addition, the production of antibodies is stimulated by the amine group present in the synthesized chitosan, which enhances the immunological response. These results are in agreement with the results reported by Miao *et al.* (2020).

As chitosan raised the antioxidant status in the liver, duodenum, and serum tissues, Cheng *et al.* (2022) discovered that chitosan-supplemented meals increased the expression of antioxidant enzymes, which in turn increased the activity of antioxidants

in the livers.

Herein, TP levels and oxidative stress index were significantly reduced in the groups treated with ChNP compared with the control group. Xu *et al*. (2020) attributed this to the strong ability of chitosan to scavenge free radicals, such as hydroxyl radicals and superoxide anions.

By controlling the activity of linked antioxidant defense enzyme systems, chitosan may lessen the concentration of active oxygen and the level of lipid peroxidation in birds and shield the body from damage caused by oxides. Chitosan can reduce oxidative stress in the liver by increasing the expression of antioxidant enzymes (Tao *et al*., 2019). Also, Khodagholi *et al*. (2010) proved that chitosan supplementation has a profound ability to prevent oxidative stress-associated damage in neurons.

In comparison with heat stressed group (control), the levels of urea and creatinine in serum in ChNP treated group exhibited a significant decrease. These findings indicated the role of ChNP in kidney protection against nephrotoxicity in stress conditions (Zaharoff *et al*., 2007; Cheng *et al*., 2022). Sudjarwo *et al*. (2017) attributed this effect to its direct action on free radicals of ChNP to prevent renal cellular damage.

Moreover, nano-chitosan improved the digestion of amino acids, Ca and P thus showing a positive effect on the broiler's growth as prior reported by Huang *et al*. (2005).

The overexpression of nutrient transporters genes with ChNP administration, glucose transporter 2 (GLUT2) and cationic amino acid transporter 1 (CAT1), is regarded as a sort of adaptive response to an improvement in nutrient digestibility, particularly lipids and protein (Zhang *et al*., 2022). These explain the remarkable increase in body weight gain and growth performance.

Moreover, the key enzymes that initiate the conversion of superoxide anion to hydrogen peroxide in cellular antioxidant processes, typically recognized as enzyme-free radical scavengers in cells, are catalase (CAT), glutathione peroxidase 1 (GPX1), and superoxide dismutase 1 (SOD1) (Vararattanavech and Ketterman 2003). Their expression was enhanced and elevated by nano-chitosan treatments, which increased the broilers' serum total antioxidant capacity (T-AOC) activity (Chang *et al*., 2023).

CONCLUSION

Supplementing drinking water with chitosan nanoparticles can enhance growth performance, nutrient ileal digestibility, and antioxidant activity by increasing the expression of the genes for amino acid transporters (CAT1 and GLUT2) and genes related to antioxidants (CAT, SOD, and GPX1). This may be the key mechanism through which ChNP attempts to promote farm animal growth.

CONFLICT OF INTEREST

The authors affirm that they have no conflict of interest.

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