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Estimation of the antibacterial activity of zinc oxide nanoparticles against induced *Clostridium perfringens* infection in broiler chickens

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ABSTRACT

A serious infection in avian species can be caused by *Clostridium perfringens* (*C. perfringens*). Because of the harmful consequences that antibiotics have on public health, reducing their usage in the animal industry is a major global concern. As an alternative to antibiotics, nanoparticles (NPs) have been used more and more recently to target bacteria. Zinc oxide (ZnO), one of these NPs, exhibited antibacterial activity even at low concentrations. Consequently, at 14 days, 75-day-old Cobb broiler chicks were separated into 5 equal groups (15 birds each divided into triplicate, 5 bird per replicate). The groups were calssified as G1, G2, G3, G4 & G5. G1 was infected with *C. perfringens* type A; G2 was infected & given zinc oxide nanoparticles (ZONPs); G3 was infected with *C. perfringens* & given doxycycline as an antibiotic; G4 received ZONPs continuously for 35 days; & G5 was the negative control. For two successive days, 4×10^8 colony forming units (CFU) of *C. perfringens* type A/mL/bird were given orally to birds in infected groups. ZONPs in the treatment groups received a total dose of 150 µg/bird. Bird performance is measured for 35 days, & data is gathered on body weight (BW), feed conversion (FC), feed conversion rate (FCR), clinical signs score, & mortalities. Moreover, immune organ indices & the microbial loads in the caecum & intestine were assessed. Remarkably, BW improvements ($P < 0.05$) were noted in ZONPs treated group & ZONPs / *C. perfringens* infected group, in contrast to those in G1. Compared to G1, treatment with ZONPs lowered the colonization of *C. perfringens* in the caeca & intestine, lessened the severity of clinical symptoms, & decreased mortality. Thymus, bursa, & spleen relative weights varied considerably ($P < 0.05$) between treatment groups. When ZONPs were administered alone or in conjunction with infection, the spleen's relative weight was considerably ($P < 0.05$) larger in treated birds than in control & antibiotic-treated birds. In conclusion, ZONPs positively affect the BW & FCR, improve the relative weight of immune organs, & can decrease the *C. perfringens* count in the intestine, positively impacting performance, general health, & gut health integrity. However, ZONPs also reduce the population of gut microbes. On the other hand, birds that received antibiotic treatment showed adverse effects on a few metrics. Therefore, more investigation into the use of ZONPs in broiler diets and their safety for the health of humans and avian species is still advised.

Introduction

The anaerobic, spore-forming, Gram-positive, rod-like bacteria *C. perfringens* causes necrotic enteritis (NE) in chickens (Baba *et al*., 1997; Lee *et al*., 2013). NE is an intestinal illness caused by *C. perfringens* in birds that is mostly brought on by microbial growth in the intestine, which produces exotoxins that destruct the gut epithelium (Dahiya *et al*., 2006). A, B, C, D, & E are the five toxinogenic kinds of *C. perfringens* based on the production of four different major toxins (Alpha, Epsilon, Beta, & Iota). It was recently concluded that an updated classification was required due to the discovery of additional toxins (NetB, Beta2, & TpeL) (Keyburn *et al*., 2008). The primary causes of NE are *C. perfringens* type A &, occasionally, type C. (Engstrom *et al*., 2003). Between two and six weeks of age, broilers are attacked by acute NE, which results in 1% daily mortality & roughly 10–40% cumulative mortality (McDevit *et al*., 2006; Cooper and Songer, 2010; Salem *et al*., 2021). According to Løvland & Kaldhusdal (1999); Hofacre *et al*. (2003), subclinical NE caused intestinal mucosal damage, which decreased digestion and absorption, depressed BWG, & elevated FCR also, it may also cause cholangiohepatitis or hepatitis. In order to cause necrosis, *C. perfringens* attaches itself to the intestinal villi, multiplies, and releases exotoxins (Shimizu *et al*., 2002). According to recent research, the incidence of *C. perfringens* in avian species was 38.3%, greater than the percentages found in earlier studies in Egypt (30 percent) (Hamad *et al*., 2020) and Pakistan (25.37%). (Haider *et al*., 2022). Reducing the use of antibiotics in cattle is a major global goal due to the harmful effects they have on public health (Marouf *et al*., 2023; Yousef *et al*., 2023). Health organizations worldwide are concerned about the growing threats posed by bacterial antibiotic resistance, which can affect human and animal health. They also want to outlaw antibiotic growth promotors in livestock (Bennett 1995). NPs are increasingly being used as a substitute to antibiotics these days (Abd El-Ghany *et al*., 2021). Using nanoparticles of mineral elements in cattle production is a promising technological advancement (Abd El-Ghany *et al*., 2021). Because of its special characteristics, which include a greater surface area, increased surface activity, a high number of surface-active centres, high catalytic efficacy, and a strong adsorbing capacity, mineral NPs have a better bioavailability (Albanese *et al*., 2012; Rajendran *et al*., 2013a). One of these NPs, zinc oxide (ZnO), has a strong antibacterial effect even at low concentrations, primarily against Gram +ve bacteria (Abd El-Haliem *et al*., 2020; Reda *et al*., 2021). This is due to the NPs' surface producing ions that destroy the bacteria's cell membrane (Shakal *et al*., 2024) & the reactive oxygen species production that results in oxidative stress and bacterial cell death (Kadiyala *et al*., 2018). Numerous studies have determined that, at the same or even lower levels, nano-zinc outperformed traditional zinc resources in terms of carcass features, productive performance, bone growth, antioxidant activity, strong growth-promoting, antimycotic, and antibacterial properties (Mohammadi *et al*., 2015; Yusof *et al*., 2019). Reduce the number of intestinal harmful microorganisms, enhance cell healing, and boost immunity (Ahmadi *et al*., 2013). Therefore, the current study aimed to assess ZnONPs' effectiveness against the experimental induction of NE in broiler chickens, paying particular attention to how this might affect the birds' immune organs & performance.

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Materials and methods

Zinc Oxide Nanoparticle's Preparation and Characterization

ZONPs' characteristics and in vitro antibacterial activity were previously described by Shakal *et al*. (2024). Five successive days, starting three days after the experimental infection and the onset of symptoms and mortality, the birds in groups G2 and G4 were given drinking water containing 2 mg ZONPs/L, respectively.

Antibiotic

The challenged bacterial strain's susceptibility to various antibiotics was tested before the drugs were chosen. Doxycycline hydrochloride 20%, 1 gram/L in water was given to the birds for five sucessive days, starting three days after the experimental infection and the onset of symptoms & mortalities.

Clostridium perfringens strain preparation

Type *C. perfringens* A strain was obtained from Cairo University's Faculty of Veterinary Medicine's Poultry Diseases Department (Salem *et al*., 2021).

Experimental design

75-day-old male Cobb broiler chicks, obtained from a commercial poultry company in Egypt, were housed on a concrete floor in deep letter system bedding with fresh wood shavings (about 10 cm thick). For 35 days, the ideal temperature, humidity, ventilation, and steady light were provided to the birds during their rearing process. Water was always available, and the birds were fed a balanced diet of starter, grower, and finisher without any additives.

At two weeks old, the chicks were randomly divided into five equal groups with 15 birds each then, each group was split up into three subgroups with five birds each.

The five experimental groups were divided as follows; G1: control positive infected group, G2: infected and zinc oxide nanoparticle-treated group at 3 days post infection (dpi), G3: infected and antibiotic-treated group at 3 dpi, G5: the blank control negative group and G4: non-infected and the zinc oxide nanoparticle-treated group all over the experimental period. Subsequently, *C. perfringens* was challenged to G1, G2, & G3 via crop gavages containing 4×10^8 CFU/ml/bird of freshly cultivated *C. perfringens* for 18 hours in phosphate buffered saline (PBS).

All birds were vaccinated against the Hitchner B1+H120 vaccine at 5 days, the LaSota vaccine at 14 days, the 228E IBDV vaccine at 18 days, and the LaSota vaccine in drinking water at 28 days.

Evaluation of the Treatment Efficacy

Every replicate's production performance was evaluated regarding live body weight (BW), feed conversion (FC), & feed conversion rate (FCR). Every week, each bird was weighed separately. FC was recorded weekly and corrected for deaths until the study's conclusion. On the days when the birds were weighed, FC was evaluated. FCR was calculated using the Timmerman *et al*. (2006) formula (g feed/g live body weight growth).

Following the experimental challenge until they were 35 days old, all birds' symptoms and deaths were recorded daily. Weekly death rates were noted, and the cumulative death rates were evaluated at the study's end.

Following the PM assessment, the gross macroscopic lesions score was recorded. Three birds from each replicate had their clinical signs scores reported, and those birds were subsequently ethically slaughtered. The six-point scale of Keyburn *et al*. (2006) & Shojadoost *et al*. (2012) was used for lesion scoring.

C. perfringens intestinal & caecal count

Every bird (3 birds/group) had its intestinal and caecal samples taken separately at 7 dpi. The samples were serially diluted in sterile PBS to a ratio of 1:100, 1:1000, & 1:10000. A 0.1 ml from each dilution was then streaked on sheep blood agar plates and Tryptose sulfite-cycloserine (TSC) agar with egg yolk. The samples were kept under anaerobic conditions for 24 hours at 37°C using anaerobic kits and Gas packs anaerobic jars. Typical *C. perfringens* (black colonies) on TSC agar or colonies with a double zone of hemolysis on blood agar plates were counted and recorded as CFU per gram. The colonies were chosen and confirmed following Harmon (1984) and Carrido *et al*. (2004).

Immune organs' relative weight (Bursa, thymus gland and spleen)

Organ weights were estimated using the formula: Related organ weight = organ weight (g) × 100 ÷ Live BW(g). Three birds per group were randomly slaughtered at 21, 28, & 35 days.

Statistical analysis

Using SPSS, 16, a one-way analysis of variance was applied to evaluate the data. When a significant difference in the means was observed, the Tukey's p test was used to contrast them. Set at $P < 0.05$, the significant result was obtained. The means ± standard error were used to represent the results.

Results

Table 1 displays the overall results of the bird performance. The BW, BWG, FC, & FCR data are provided. At weeks number 2, 3, 4, & 5, there were significant ($P < 0.05$) differences in the BW & FC between the negative control birds, the infected controls, & the ZONPs supplied groups. However, at weeks number 3 & 4, there were no differences in BW, BWG, or FCR between the different groups at different criteria. Interestingly, when comparing the body weights of the ZONPs treated group & ZONPs

Table 1. Effect of zinc oxide nanoparticle and antibiotic treatments on body weight, body weight gain, feed intake, and feed conversion ratio in infected broiler.

Group	Body weight at age /kg					Body weight gain/kg	Cumulative FC (kg)	Cumulative FCR
	1st week	2nd week	3rd week	4th week	5th week	Total		
G1	0.15±.004	0.40±.009	$0.81±.018^a$	$1.11±.04^b$	$1.65±.05^b$	$1.48±.008^d$	$2.62±.008^b$	$1.77±.008^a$
G2	0.16±.005	0.39±.006	$0.82±02^a$	$1.24±.03^a$	$1.84±.05^{ab}$	$1.64±.018^c$	$2.09±.003^c$	$1.28±.008^c$
G3	0.16±.004	0.41±.007	$0.58±.03^b$	$1.24±.03^a$	$1.85±.15^{ab}$	$1.68±005^c$	$2.65±.028^{ab}$	$1.57±.008^b$
G4	0.156±.003	0.41±.015	$0.82±.02^a$	$1.22±.03^{ab}$	$2.16±.09^a$	$1.93±.010^a$	$2.50±.018^b$	$1.29±.014^c$
G5	0.156±.004	0.42±.006	$0.79±.02^a$	$1.13±.03^{ab}$	$2.13±.03^a$	$1.84±.008^b$	$2.72±.025^a$	$1.47±.008^b$
P value	0.29	0.26	0	0.02	0.00	0	0	0

3 replicates/group (15birds/group). Means (Mean ±SE) with different lowercase letters in the same column indicate significant differences at $P < 0.05$. SE: standard error.

/ Clostridium infected groups to those of the positive birds, substantial (P < 0.05) improvements were noted. The BW & BWG increased linearly with age, with G4 & G5 being the greatest values at all experimental weeks as compared to G1.

The effects of multiple treatments on the clinical lesion ratings of three birds per experimental group. Throughout the whole trial period, none of the G5 or G4 birds displayed any clinical signs (clinical lesion score = 0). Bacterial infection was visible in the infected and combined treated subgroups at 21–28 days of age (G2 & G3). When comparing the clinical lesion ratings of the birds in the infected duplicates to the positive controls, we observed that administering ZONPs (G2) treatment regimens led to a substantial decrease. The birds treated with ZONPs (G2) showed less severity of clinical signs than groups challenged with *C. perfringens* G1, G2, & G3.

Results in Figure 1 demonstrated a significant reduction in the severity of clinical signs relative to infected groups, which displayed a normal appearance of intestinal & mesenteric blood vessels.

The data show how did the different treatments affected the survival rates of birds across all experimental groups. It has been demonstrated that there were no mortality in either the ZO-NPs-infected or negative control groups over the whole trial period. In the meantime, the proportions of the total deaths of birds in the subgroups that were treated with ZO-NPs (G2), antibiotics (G3), and infection (G1) were 40 % (6/15), 20 % (3/15), and 6.6% (1/15), respectively.

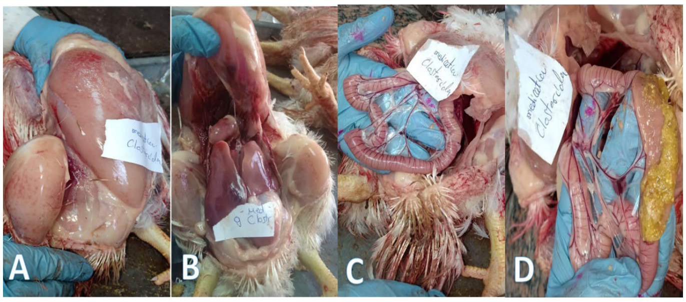

Fig. 1. Postmortem examination of birds experimentally infected and antibiotic treated birds showing A: hemorrhages on breast and thigh muscles, B: hemorrhages on liver, C: enteritis with engorgement of mesenteric blood vessels, D: opened intestine containing undigested feed particles mixed with gases.

Table 2 contains the intestinal and caecal *C. perfringens* count. The acquired results demonstrated that, as compared to the infected group, the ZONPs-supplied infected group, the antibiotic-treated infected group, and the ZONPs-only group all exhibited considerably lower cecal *C. perfringens* counts (P = 0.000). (G1). The study revealed that the intestinal *C. perfringens* count was lower in the G2, G3, & G4 groups compared to the control group, which had a considerably (P < 0.05) greater count.

Table 2. Effect of ZO- NPs on broiler chicken cecal and intestinal *C. perfringens* counts (colonies) after 7-day post infection

Groups	Caecal	Intestinal
G1	11.61 ±.33[a]	10.09±.66[a]
G2	8.23±.36[b]	7.49±.35[b]
G3	8.80±.27[b]	7.61±.53[b]
G4	8.23±.36[b]	7.49±.35[b]
G5	9.34±.34[b]	9.53±.33[ab]
P value	0	0.01

3 replicates/group (15birds/group). Means (Mean ±SE) with different lowercase letters in the same column indicate significant differences at P < 0.05. SE: standard error.

The findings shown in Table 3 suggest that administering ZONP supplements impacts the relative weights of immunological organs, particularly the spleen. Significant differences were seen in the relative weights of the spleen, bursa, and thymus across the three treatment groups (P < 0.05). Compared to the control and other treatments, the spleen's relative weight in birds treated with ZONPs, either alone or in conjunction with infection, was considerably (P < 0.05) higher.

However, the proportional weight of the thymus and bursa in birds exposed to ZONPs was considerably (P < 0.05) greater than that of the control and other treatment groups.

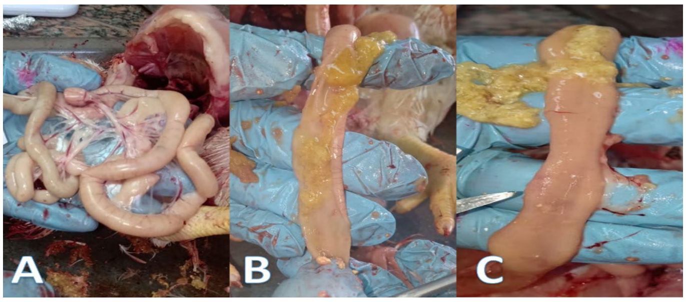

Fig. 2. Postmortem examination of birds experimentally infected and nanoparticles treated birds showing A: normal intestinal and mesenteric blood vessels appearance, B and C: Opened intestine containing undigested feed particles with apparently normal intestinal mucosa.

Discussion

Major intestinal infections, including clinical and subclinical forms of NE, harm the profitability of the broiler production process (Bansal *et al*., 2021; Salem *et al*., 2021). Subclinical NE may cost as much as 5 cents per bird, according to a research by Van der Sluis (2000), and NE outbreaks can cost the global broiler industry up to $2 billion yearly. Zinc oxide nanoparticles (NPs) are a viable substitute for traditional dietary supplements in birds, allegedly having a greater effect than inorganic and organic versions (Gopi *et al*., 2017). According to earlier research, nano-zinc outperformed conventional zinc resources regarding productive performance, bone growth, carcass traits, antioxidant effect, as well as antibacterial & antimycotic properties. It also possesses growth promoter

Table 3. Effects of ZONPs supplementation on carcass and immune organs' relative weight (g) chicks at different age at 3rd week, 4th week, and 5th week old broilers (Mean ±SE).

Groups	Bursa of Fabricius			Thymus			Spleen		
	3rd week	4th week	5th week	3rd week	4th week	5th week	3rd week	4th week	5th week
G1	0.130±0.001[b]	0.134±0.001[a]	0.086±0.0003[d]	0.867±0.004[c]	0.726±0.002[b]	0.706±0.000[d]	0.065±0.000	0.063±0.002[b]	0.068±0.001[b]
G2	0.133±0.001[b]	0.103±0.000[c]	0.080±0.0003[c]	0.502±0.001[a]	0.568±0.006a	0.525±0.003[b]	0.072±0.001	0.071±0.001[c]	0.126±0.003d
G3	0.131±0.001[b]	0.136±0.000[a]	0.0570±.0003[a]	0.867±0.004[c]	0.922±0.001[d]	0.487±0.002[a]	0.065±0.000	0.052±0.001[a]	0.054±0.002[a]
G4	0.113±0.001[c]	0.119±0.001[b]	0.090±0.0003[e]	0.769±0.001[b]	0.747±0.001[c]	0.489±0.001[a]	0.082±0.010	0.098±0.001[d]	0.129±0.001d
G5	0.137±0.001[a]	0.134±0.000[a]	0.076±0.0003[b]	0.928±0.001[d]	0.721±0.001[b]	0.674±0.009[c]	0.069±0.001	0.074±0.002[c]	0.077±0.001[c]
P value	0	0	0	0	0	0	0.13	0	0

3 replicates/group (15birds/group). Means (Mean ±SE) with different lowercase letters in the same column indicate significant differences at P < 0.05. SE: standard error.

capabilities, which can lower the growth of intestinal pathogenic bacteria, increase cell repair, and boost immunity (Zhao *et al*., 2014; El-Katcha *et al*., 2017; Yusof *et al*., 2019). Apart from its role in development, metabolism, and physiological and biosynthetic processes in the bodies of birds, zinc also performs additional important roles (Cesur *et al*., 2005).

The investigated broiler performance characteristics, as presented in Table 1, indicate that the birds treated with ZONPs (G4) exhibited significantly greater BW (P = 0.001) & BWG (P < 0.000) compared to the control birds (G1 & G5). Furthermore, noteworthy enhancements were documented in the FCR of birds (G2 & G4) that were administered ZONPs (P < 0.000). These findings might have improved broiler growth performance; the performance improvement might have resulted from zinc's beneficial effects on nutrient absorption and digestion in the gut and its increased bioavailability in the form of NPs (Hussan *et al*., 2022). Additionally, birds treated with nano zinc exhibit improved performance because of increased absorption of zinc nanoparticles in the gut and faster intestinal cell diffusion due to the particle size of nano zinc. Our findings corroborate those of Zhao *et al*. (2014), who observed that birds in the concentration of 20 and 60 mg dietary ZONPs groups had considerably higher BWG & superior FCR after two weeks. Because zinc is a crucial microelement that affects bird performance, carcass features, and meat quality of broilers exposed to heat stress, using zinc oxide nanoparticles could also help lessen the negative effects of heat stress and increase broiler production (Rajendran *et al*., 2013b). G2, G4, & FCR have higher BW & FCR.

The increased BW in the group that received zinc oxide NPs may be due to organic minerals, which are an excellent way to provide broilers with more minerals without raising the levels of minerals in their feed, as suggested by Abdallah *et al*. (2009).

The impact of several therapies on the bird's clinical macroscopic lesion score. Throughout the whole experimental period in this investigation, neither the birds in the ZONPs exchanged group (G4) nor the negative control group (G5) displayed any clinical indications (clinical score = 0). In contrast, ruffled feathers, an overall depressive state, and orange, foamy droppings were noted as symptoms in G1 and G3. Comparable outcomes have been shown in chickens with experimental *C. perfringens* infection (Awaad *et al*., 2019). When ZONPs (G2) were administered in the current study, it was observed that the clinical sign scores of the birds in the infected subgroups were significantly lower than those of the positive controls. birds treated with antibiotics and experimentally afflicted birds' PM Figure 2 depicts haemorrhages on the muscles of the breast and thighs, subcapsular hemorrhages on the liver, enteritis with engorgement of the mesenteric blood vessels, and undigested feed particles mixed with gases in the intestine. The experimentally infected and nanoparticle-treated birds' PM examination in Figure 2 revealed normal appearance of the intestinal and mesenteric blood vessels and normal intestinal mucosa. This could be attributed to the birds' improved organ structure, higher Zn retention in the hepatic tissues post-uptake, and NPs' improved zinc bioavailability (Yusof *et al*., 2019).

The mortlity rates for G1 and G2 were 40% & 6.6%, respectively. It has been shown that NE causes a cumulative mortality of 6.6% to 40% in broiler chickens. Due to its high morbidity & mortality rates, *C. perfringens* is one of the most common illnesses in birds globally, resulting in unexpected and significant financial losses of billions of dollars annually (Wade and Keyburn 2015). It has been linked to illnesses affecting 55.9% of Egyptian chickens and poultry products (Shaaban *et al*., 2017). The lower mortality rate (7.41 %) reported by Awaad *et al*. (2019) in chickens challenged experimentally with *C. perfringens* could be attributed to variations in the bacterial strain, dose, challenge route, or experimental conditions. One possible explanation for the 6.6% decrease in mortality in the G2:infected & ZONPs provided group is the antibacterial effect of NPs on *C. perfringens* infection.

In the present investigation, the doxycycline-treated birds displayed subcapsular haemorrhage in the liver and haemorrhages in the muscles (Figure 2). These results were consistent with those reported by Goma *et al*. (2018), who discovered that doxycycline overdose had a negative effect on quail performance and caused lipidosis in liver tissues, cerebral blood vessel congestion, renal lesions, and hemorrhages.

The study's findings support the idea that giving broiler chicks ZONP water supplements led to a notable increase in BW & better FCR when compared to control chicks. The current findings corroborated those of Hussan *et al*. (2022), who found that adding 2.5 ppm of nano ZnO to the broiler feed resulted to noticeably greater BWG, FI, & better FCR. The current findings are consistent with those of El-Haliem *et al*. (2020), who examined the effects of dietary zinc oxide at different concentrations (20, 40, 60, 80, & 100 mg/kg feed) on the growth performance and financial efficiency of Cobb broiler chicks over six weeks and their findings indicated that birds fed 40 mg ZONPs had superior LBW, TBWG, & FCR values when compared to the other groups.

As shown in Table 2, the cecal and intestinal counts of *C. perfringens* at 7 dpi for G2, G3, & G4 in this study were lower (P = 0.000) than those for G1. In addition, intestinal *C. perfringens* numbers were considerably lower in G2 and G3 compared to G1 & G4 (P < 0.005). Regarding the intestinal & cecal *C. perfringens* counts, the applied ZONP concentration had the strongest antibacterial impact. Some scientists hypothesised that increased permeability, which interferes with normal transport through the cell membrane, is the cause of bacterial cell death (Auffan *et al*., 2009). The primary determinants of nanoparticles' antibacterial effects are their surface area & concentration (Arabi *et al*., 2012). The greater the surface area available for bacterial contact & the stronger antibacterial effect of NPs, the lower their particle size (Adams *et al*. 2006).

Our findings in Table 3 demonstrate a substantial variance (P <0.005) in the experimental broiler chickens' relative immune organ weight. Additionally, at the 3rd & 4th weeks, a significant difference was observed in all immune organ indices across all treated groups. The influence of ZONPs on the relative weight of the internal organs was consistent with the findings of Ahmadi *et al*. (2013) & Sagar *et al*. (2018), who reported a significant improvement in the relative weight of the spleen, bursa, & thymus in broilers after ZONPs were added to their diet. This could be explained by the antibacterial properties of ZONPs, which reduce the burden of harmful microorganisms & boost gut immunity in addition to zinc's function in boosting immunity & promoting overall health (Sahoo *et al*., 2014).

Conclusion

Our research leads us to the conclusion that applying ZONPs can improve overall health, improve gut health, reduce *C. perfringens* counts in broiler chickens and have a positive effect on birds' productivity; additionally, ZONPs have positive effects on BW & FCR and raise the relative weight of immune organs. On the other hand, using antibiotics in those infected had a adverse impact on certain measures. Therefore, more research is still needed to determine the safety of ZONPs, their impact on other microorganisms, & how to use them in broiler rations.

Conflict of interest

The authors declare that they have no conflict of interest.

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