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# Evaluation of Lysozyme Supplementation on Antioxidant Status, Immune Gene Expression, and Amelioration of Necrotic Enteritis in Broiler Chickens

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**Abstract**

THIS study investigated the efficacy of lysozyme supplementation, alone and in combination with amoxicillin, in mitigating the detrimental effects of *Clostridium perfringens* and *Eimeria* co-infection in broiler chickens. One-hundred-twenty day-old broiler chicks were randomly divided into six groups: uninfected control, infected control, prophylactic lysozyme, therapeutic lysozyme, therapeutic amoxicillin, and therapeutic lysozyme + amoxicillin. Birds were challenged with *Eimeria* and *C. perfringens*. Lysozyme supplementation significantly improved growth performance, reduced oxidative stress, normalized cortisol levels, and enhanced haematological parameters (including erythrocyte counts and leukocyte profiles). Serum lysozyme activity was significantly elevated in lysozyme-treated groups. The G6 group (lysozyme + amoxicillin) exhibited the most pronounced improvements in all assessed parameters. This group demonstrated the lowest *C. perfringens* counts, reduced oxidative stress markers (MDA, NO), increased antioxidant levels (CAT, SOD), and normalized cortisol and protein levels. Quantitative PCR analysis revealed increased *GSH* gene expression and decreased *IL*-6 gene expression in the G6 group. All treatment groups showed increased *IL-1β* and *IgA* gene expression, while *IgY* gene expression was upregulated in the G3 (prophylactic lysozyme) and G6 groups. These findings demonstrate that lysozyme supplementation, particularly in combination with amoxicillin, effectively mitigates the detrimental effects of *C. perfringens* and *Eimeria* co-infection in broiler chickens. The observed improvements can be attributed to enhanced immune responses, improved antioxidant status, normalized physiological parameters, and reduced bacterial burden, highlighting the potential of lysozyme as a valuable tool for enhancing broiler health and optimizing production.

**Keywords:** *Clostridium perfringens*; Co-infection; Feed Additive; Intestinal Microbiota; Mucosal Immunity, Oxidative Stress; Poultry Health.

## Introduction

Necrotic enteritis (NE) poses a substantial economic threat to the broiler chicken industry globally. This disease, primarily induced by the proliferation of the opportunistic bacterium *Clostridium perfringens* within the intestinal tract, is characterized by severe intestinal inflammation and necrosis [1]. While *C. perfringens* is a common inhabitant of the poultry gut, factors such as coccidial infections, dietary imbalances, and stress can disrupt the delicate intestinal microbiota, leading to dysbiosis and the subsequent rapid multiplication of this pathogen [2]. The resulting damage to the intestinal lining severely compromises nutrient absorption, ultimately resulting in reduced growth rates, impaired feed conversion

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efficiency, increased mortality, and significant economic losses for poultry producers [3].

Historically, the control and prevention of NE have heavily relied on the prophylactic and therapeutic use of antibiotics. However, the escalating global concern over antimicrobial resistance, including the emergence of antibiotic-resistant strains of *C. perfringens*, necessitates the urgent exploration and development of alternative, sustainable strategies for maintaining gut health and controlling this economically devastating disease [4]. In this context, natural feed additives have garnered considerable research interest, with lysozyme emerging as a promising candidate due to its multifaceted beneficial properties [5–7].

Lysozyme, a naturally occurring antimicrobial enzyme, is a key component of the innate immune system found in various biological fluids and tissues, including egg white, milk, saliva, tears, and the intestinal mucus of animals, including poultry. This enzyme exerts its antimicrobial action primarily by catalyzing the hydrolysis of the β-(1,4) glycosidic bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), the main structural components of the peptidoglycan layer in the cell walls of Gram-positive bacteria, leading to cell lysis and death [8]. Beyond its direct bactericidal activity, lysozyme exhibits a range of other beneficial effects relevant to gut health and overall animal performance. Studies have demonstrated its ability to modulate the gut microbiota composition, promoting a more balanced and beneficial microbial profile [9]. Furthermore, lysozyme has been shown to possess anti-inflammatory properties by interacting with immune cells and influencing the production of cytokines, thereby potentially mitigating the intestinal inflammation associated with NE [10]. In addition, lysozyme can enhance the integrity of the intestinal barrier, reducing the translocation of harmful bacteria and toxins into the bloodstream [11]. Its potential to boost the innate immune response by activating macrophages and promoting the production of immunoglobulins further contributes to its protective effects against enteric pathogens [12].

Given these promising attributes, this study aimed to investigate the efficacy of dietary lysozyme supplementation in mitigating the detrimental effects of experimentally induced NE in broiler chickens. Specifically, we evaluated the impact of lysozyme on disease severity by monitoring mortality rates, clinical signs, and intestinal lesion scores and antioxidant status by assessing key antioxidant parameters such as malondialdehyde (MDA), superoxide dismutase (SOD), and catalase activity. Additionally, we investigated the influence of lysozyme on the host immune response by analyzing the expression of immune-related genes, including cytokines and immunoglobulins.

This study seeks to provide novel and comprehensive insights into the mechanisms underlying the beneficial effects of lysozyme in alleviating NE in broiler chickens, offering further evidence for its potential as a valuable and sustainable alternative to antibiotics for improving gut health, enhancing bird performance, and reducing economic losses in the poultry industry.

## Material and Methods

### *Ethical Acceptance*

The principles outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and the regulations set by the Assiut University Animal Care and Use Committee (Approval No. 06/2024/0212) were strictly adhered to during all animal procedures.

### *Birds*

From a commercial hatchery, 120 one-day-old Ross broiler chicks were acquired. The chicks were examined and found to be free of *Clostridium perfringens* and *Eimeria* species using conventional PCR and bacteriological methods [8]. At Assuit University in Egypt, the Experimental Animals Research Unit of the Faculty of Veterinary Medicine kept the birds in isolators with regulated environmental conditions (temperature: 32 ± 2°C, humidity: 60–70%). The birds were provided with unlimited access to water and a commercially balanced food.

### *Preparing the Inoculum*

The *C. perfringens A* reference strain was cultivated in cooked pork broth and incubated in an anaerobic environment at 37°C for 24 to 48 hours using a GasPak device.

### *Bird Challenge*

One dose of $1\times10^4$ sporulated oocysts of a reference strain of *Eimeria* species was orally administered to all infected groups at the age of 14. As mentioned by Gharib-Naseri et al. [13], on day 19, the same groups were orally challenged with 1

mL ($10^8$ CFU/mL) of a *C. perfringens* suspension prepared according to the McFarland standard via oral gavage.

*Experimental Design*

A total of 120 one-day-old broiler chicks were randomly divided into six groups (n=20/group):

Group 1 (Control): Uninfected and untreated.

Group 2 (C+ve): Infected with *Eimeria* and *C. perfringens*.

Group 3: Prophylactic treatment with lysozyme (0.5 mL/L) in drinking water from day 9 to 13.

Group 4: Therapeutic treatment with lysozyme (0.5 mL/L) in drinking water from day 20 to 24.

Group 5: Therapeutic treatment with amoxicillin (20 mg/kg) in drinking water from day 20 to 24.

Group 6: Combination treatment with amoxicillin (20 mg/kg) and lysozyme (0.5 mL/L) in drinking water from day 20 to 24.

*Sample Collection and Analysis*

Throughout the 28-day experiment, chickens were monitored for clinical signs. At the end of the trial, all birds were weighed individually, and average body weight was calculated for each group. Five birds per group were then euthanized, and their intestines were collected for examination. Necrotic enteritis lesions were scored using the 0-4 scale described by Prescott et al. [14]. This scoring system is defined as follows:

0: No gross lesions, healthy gut appearance.

1: Gray, friable, or thin-walled intestinal segments.

2: Focal necrosis with thin walls, gray appearance, and slight gas production.

3: Large, thin-walled necrotic areas, gas-filled gut, and small blood spots.

4: Major necrotic lesions, significant bleeding, and abundant gas in the intestine.

**Cecal Contents:** At the end of the experiment (day 28), cecal contents were collected from all groups aseptically. Serial dilutions of the homogenized cecal contents were plated on reinforced clostridium agar (RCM) and incubated anaerobically at 37°C for 48 hours. Colony-forming units (CFU) of *C. perfringens* were counted.

**Blood Collection:** On day 28, blood samples (approximately 2 mL) were obtained from each bird in each group via wing vein puncture using sterile syringes containing 7.2 mg of dipotassium ethylenediaminetetraacetic acid ($K_2$EDTA) per mL of blood as an anticoagulant**.**

For hematological examination (erythrogram and leucogram), whole blood samples collected with $K_2$EDTA were analyzed using an automatic blood cell counter (Sysmex KX-21N Automatic Hematology Analyzer)**.** The following hematological parameters were carried out: Red blood cell count (RBC), Hemoglobin **(**Hb) concentration, Packed cell volume (PCV), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), White blood cell count (WBC), and differential leukocyte count (neutrophils, lymphocytes, monocytes, and eosinophils)**.** Blood smears were prepared and stained with Giemsa stain for manual differential leukocyte counts to confirm the automated results.

*Biochemical Analyses:* Serum biochemical parameters were analyzed to assess the impact of *C. perfringens* infection and the effects of the treatments as follow:

*Oxidative Stress and Malondialdehyde (MDA):* The levels of MDA, a byproduct of lipid peroxidation, were measured using the thiobarbituric acid reactive substances (TBARS) assay, a conventional technique for evaluating lipid peroxidation.

*Antioxidant Enzymes:* Catalase (CAT) activity was measured by tracking the breakdown of hydrogen peroxide using spectrophotometric measurements. The activity of superoxide dismutase (SOD) was tested using the nitroblue tetrazolium (NBT) reduction method, which gauges the enzyme's capacity to prevent NBT reduction by superoxide radicals.

*Hormones:* Levels of serum cortisol, a crucial stress hormone, were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cat. No. K7430-100).

*Other Parameters:* The Biuret method, a reliable colorimetric test for protein quantification, was utilized to estimate the total protein content [15]. Nitric oxide (NO) levels were determined using the Griess reaction, a colorimetric technique for identifying nitrite, a stable metabolite of NO [16].

*Lysozyme Activity:* An ELISA kit (Catalog Number: MBS725718) was used to measure serum lysozyme activity, which serves as an indication of innate immunity, following the manufacturer's instructions.

*Gene Expression Analysis:* Total RNA was extracted from intestinal mucosal tissues, the liver, and the spleen using the RNeasy Mini Kit (Qiagen, Germany) as per the manufacturer's recommendations. The amount and quality of RNA were evaluated using a spectrophotometer.

A StepOne Real-Time PCR System (Applied Biosystems) with SYBR Green detection, set up in the Biotechnology Unit of the Animal Health Research Institute at the Zagazig Branch in Egypt, was utilized to perform quantitative real-time PCR (qPCR). The expression levels of target genes for cytokines, immunoglobulins, and antioxidant enzymes were evaluated. The housekeeping gene β-actin was used as an internal control for normalization. Each reaction consisted of 10 microliters of the 2x HERA SYBR® Green RT-qPCR Master Mix (Willowfort, UK), 0.5 microliters of forward and reverse primers, 1 microliter of RT Enzyme Mix (20X), 3 µL of water, and 5 µL of RNA template. After a 15-minute initial denaturation at 95°C, the PCR was cycled 40 times: 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. The $2^{-\Delta\Delta Ct}$ method was employed to determine relative gene expression levels. ΔCt represents the variance in cycle threshold (Ct) values between the target gene and the β-actin housekeeping gene, while ΔΔCt indicates the variance in ΔCt values between the experimental groups and the control group [17,18].

*Statistical analysis*

The program used to analyze all results was SPSS Inc., version 26 (IBM Corp., Armonk, NY, USA). Shapiro-Wilk's and Levene's tests were employed to evaluate the homogeneity and normality of the experimental groups, respectively. Experimental trial data were presented as means ± standard error of the mean (SEM), and Tukey's tests and one-way ANOVA were conducted to determine if there were significant differences between the mean values. Variations were considered statistically significant when the *p*-value was less than 0.05. GraphPad Prism software (San Diego, USA) was utilized to generate all graphs.

## Results

*Body Weight and Clinical Observations*

At 28 days of age, broiler chicks in the lysozyme-treated groups (G4 and G6) exhibited significantly higher average body weights compared to the infected control group (G2) (Table 2). No significant difference in body weight was observed between the prophylactic lysozyme group (G3) and the amoxicillin group (G5).

Prior to *C. perfringens* challenge, no apparent clinical signs were observed in any group. Following challenge, all challenged groups displayed signs of depression, dullness, and wet, brownish droppings. These symptoms were most pronounced in the infected control group (G2), which also exhibited decreased appetite and undigested feed in the droppings. Groups G6, G4, and G5 showed improvement in these symptoms following treatment, with G6 showing the most pronounced recovery. Average body weights (in grams) at 28 days are presented in Table 2.

*Impact of Treatments on C. perfringens Counts*

The impact of various treatments on *C. perfringens* counts in experimentally infected birds is illustrated in Fig. 1. All treatment groups exhibited a significant ($p < 0.0001$) reduction in *C. perfringens* counts (up to 5.04 Log10 CFU/g) compared to the infected control group (G2, 5.86 Log10 CFU/g). The *C. perfringens* levels in the treatment groups, including the negative control group (G1) and the G6 group (lysozyme + amoxicillin combination), did not differ significantly.

*Serum Biochemistry*

*Lysozyme Activity*

The serum lysozyme activity, as depicted in, showed distinct patterns across the experimental Fig. 2 groups. The positive control (G2) group exhibited a significantly higher serum lysozyme activity compared to the negative control (G1) group ($p < 0.05$). In the prophylactic lysozyme therapy group (G3), serum lysozyme activity was notably lower than in the G2 group ($p < 0.05$). Similarly, therapeutic treatments with lysozyme (G4) and amoxicillin (G5) resulted in markedly reduced serum lysozyme activity compared to the G2 group ($p < 0.05$). Contrary to the initial expectation, serum lysozyme activity was significantly higher in the lysozyme-treated groups (G4, G3, and G6) than in the control groups (G2 and G1), with the G4 group

exhibiting the highest serum lysozyme activity ($p < 0.05$)

*Oxidant and Antioxidant Parameters*

Serum levels of reduced superoxide dismutase (SOD), catalase (CAT), nitric oxide (NO), and malondialdehyde (MDA) are summarized in Table 3. MDA and NO levels were noticeably higher in the control infected group (G2) than in any of the other groups, suggesting increased oxidative stress. In contrast, the CAT and SOD levels in the G2 group were noticeably lower than those in the other groups. Interestingly, when compared to the lysozyme-treated group after infection (G3) and the amoxicillin-treated group (G5), the lysozyme-treated group before infection (G4) and the amoxicillin + lysozyme-treated group (G6) showed noticeably better antioxidant status.

*Other Serum Biochemical Parameters*

Table 4 summarizes the results of serum cortisol and total protein levels. The G2 group exhibited significantly elevated cortisol levels and significantly lower total protein levels compared to all other groups. Conversely, both the G4 and G6 groups showed significant improvements in cortisol and total protein levels compared to the G3 and G5 groups.

*Haematological Findings*

*Erythrogram*

Table 5 presents the erythrogram parameters. The G2 group exhibited significant reductions in hemoglobin (Hb) and mean corpuscular volume (MCV) ($P < 0.05$), indicative of microcytic hypochromic anemia**.** In contrast, the G3 and G4 groups showed marked and statistically significant ($P < 0.05$) improvements in erythrogram parameters (Hb and MCV) compared to the G5 and G6 groups. The G5 group exhibited microcytic normochromic anemia**,** characterized by significantly reduced MCV ($P < 0.05$) but non-significantly altered mean corpuscular hemoglobin concentration (MCHC) compared to the control group (G1). The G6 group exhibited macrocytic hyperchromic anemia**,** characterized by a significantly increased MCV ($P < 0.05$) and a significantly increased mean corpuscular hemoglobin concentration (MCHC) of 39.31 ± 1.2 g/dL ($P < 0.05$).

*Leucogram*

Table 6 also summarizes the leucogram parameters. The G2 group exhibited a significant decrease ($P < 0.05$) in white blood cell (WBC) count compared to the other groups, indicating leucopenia. The G3 and G4 groups showed a marked and statistically significant ($P < 0.05$) improvement in the leucocyte picture compared to the G2 group, with WBC counts returning closer to the control (G1) levels.

Notably, the G5 and G6 groups presented with relative percentages for the differential leukocyte count (DLC). Specifically, G5 exhibited heteropenia (decreased heterophil percentage), lymphocytosis (increased lymphocyte percentage), and monocytosis (increased monocyte percentage). Similarly, G6 also showed heteropenia, lymphocytosis, and monocytosis based on the relative percentages of these cells.

All groups exhibited monocytosis compared to the G1 group. The most pronounced monocytosis, based on absolute counts ($x10^3/\mu l$)**,** was observed in the G2 group (Table 6).

*Quantitative PCR Findings*

Gene expression analysis revealed significant alterations in response to the different treatment groups.

***GSH*:** Compared to the G2 group, the G6 group (lysozyme + amoxicillin) showed a significant 1.85-fold increase in *GSH* gene expression ($p = 0.011$) as shown in Fig. 3. There were no noticeable changes between the G1, G5, and G3 groups.

**Cytokines:** The G6 group's *IL-6* expression was 1.57 times lower than the G2 group's (7.05 times lower) ($p = 0.006$). The G3, G4, and G5 groups did not differ significantly from one another (Fig. 4A).

All treatment groups showed a significant increase in *IL-1β* expression compared to the G2 group ($p < 0.0001$), with the G6 group showing the highest expression (1.89-fold, Fig. 4B).

The G3 (prophylactic lysozyme) and G6 (lysozyme + amoxicillin) groups exhibited significantly higher levels of *IL-10* expression than the G2 group ($p = 0.001$), with the G6 group showing the highest expression (1.19-fold, Fig. 4C).

**Immunoglobulins:** The analysis of *IgA* and *IgY* gene expression is presented in Fig.5. When comparing the G6 group (lysozyme + amoxicillin) to the G2 group, there was a significant increase ($p =$

0.003) in *IgA* gene expression, which rose by 0.8 times from a baseline of 0.31 times. Fig. 5A indicates that there was no noticeable difference in *IgA* expression between the G6 and G1 groups. Similarly, the *IgY* gene expression in the G6 group was significantly higher ($p$ = 0.001) than that of the G2 group, increasing by 0.8 times from a baseline of 0.2 times. Fig. 5B shows that there was no discernible difference in *IgY* expression between the G6 and G1 groups.

## Discussion

Broilers have a remarkable capacity for converting feed into meat, making them highly efficient agricultural animals [19–21]. However, optimal broiler performance, encompassing parameters such as body weight, weight gain, feed conversion ratio, and protein efficiency ratio, is critically dependent on intestinal health and the delicate balance of the gut microbiota. A robust gut ecosystem plays a pivotal role in various physiological processes, including mucosal immunity, nutrient absorption, and overall gut development [22–26].

Lysozyme (LYZ), a natural antimicrobial enzyme, has shown promise in enhancing both broiler performance and gut health in previous studies [27]. The current study further supports these findings, demonstrating that supplementation with both LYZ and amoxicillin significantly improved key performance indicators compared to the control group. These observations underscore the potential of dietary LYZ supplementation in optimizing broiler production while promoting gut health and overall animal well-being.

Lysozyme supplementation offers a promising approach to modulating gut microbiota and improving broiler performance. It has been shown to reduce populations of harmful bacteria, such as *Clostridium perfringens*, while potentially fostering the growth of beneficial bacteria. This improved gut microbial balance can enhance nutrient digestibility and absorption, leading to better growth performance, consistent with previous findings that lysozyme supplementation improves feed conversion ratio and overall growth in broilers [27,28].

In this study, all treatment groups exhibited a significant reduction in *C. perfringens* counts compared to the infected control group (G2), demonstrating the efficacy of the treatments in controlling the infection. This reduction in bacterial load likely results from the combined antibacterial activity of amoxicillin and the immunomodulatory effects of lysozyme. Lysozyme's mechanisms of action include direct lysis of bacterial cell walls and modulation of the immune response, both of which likely contributed to the observed decrease in *C. perfringens* counts [29].

The significant increase in serum lysozyme activity observed in the lysozyme-treated groups (G4, G3, G6) provides strong evidence for the efficacy of lysozyme supplementation in enhancing the bird's innate immune response. As a natural antimicrobial enzyme, lysozyme directly lyses bacterial cell walls. Increased serum lysozyme activity can contribute to more efficient bacterial clearance, thereby reducing the bacterial burden and mitigating the severity of infections. Several studies have demonstrated the ability of dietary lysozyme supplementation to enhance serum lysozyme activity in poultry [30].

Furthermore, lysozyme supplementation has been shown to modulate the gut microbiota, contributing to improved gut health. Indeed, lysozyme supplementation significantly reduces the abundance of harmful bacteria [31]. Park et al. [32] found that lysozyme inclusion significantly increased the abundance of beneficial bacteria, such as *Lactobacillus*, while reducing the abundance of *E. coli* in the ileum of pigs. These findings suggest that lysozyme can suppress the growth of harmful bacteria like *Clostridium perfringens* and *Total coliforms*, while simultaneously supporting the growth of beneficial bacteria like *Lactobacilli*. This balanced modulation of the gut microbiota ultimately contributes to healthier intestinal development in broiler chickens.

The clinical observations further support the beneficial effects of lysozyme. Following *C. perfringens* challenge, all challenged groups exhibited signs of illness, including depression, dullness, and wet, brownish droppings. These symptoms were most severe in the infected control group (G2), consistent with the expected pathology of necrotic enteritis. The presence of undigested feed in the droppings of this group suggests impaired nutrient absorption due to intestinal damage. Importantly, the lysozyme-treated groups (G4 and G6), along with the amoxicillin group (G5), showed improvement in these clinical signs, with the combination treatment (G6) exhibiting the most pronounced recovery. This suggests that lysozyme,

particularly in combination with amoxicillin, effectively mitigated the negative effects of the *C. perfringens* challenge. The improved clinical status of the treated groups likely contributed to their better growth performance. The synergistic effect observed in the combination group (G6) suggests that lysozyme and amoxicillin may work through complementary mechanisms to combat the infection and promote gut health. Amoxicillin directly targets the bacteria, while lysozyme likely enhances the host's immune response and contributes to bacterial lysis, creating a multi-pronged approach to combating the pathogen.

The elevated levels of malondialdehyde (MDA) and nitric oxide (NO), coupled with decreased levels of catalase (CAT) and superoxide dismutase (SOD) in the G2 group (infected with *C. perfringens* and coccidia), strongly suggest increased oxidative stress in these birds. *C. perfringens* infection can induce oxidative stress through various mechanisms, including the release of reactive oxygen species (ROS) by activated immune cells and the disruption of cellular antioxidant defense systems [33–35]. Elevated MDA levels are a hallmark of lipid peroxidation, a key indicator of oxidative damage [36]. While NO production can have both beneficial and detrimental effects depending on the level, excessive NO production can contribute to oxidative stress [37].

In line with these findings, the significant improvements observed in MDA, NO, CAT, and SOD levels in the groups supplemented with lysozyme (LYZ) suggest a protective role against oxidative stress. Notably, lysozyme supplementation has been shown to play a pivotal role in stimulating both cellular and humoral immunity [29,38]. Furthermore, studies by Jiménez-Saiz et al. [39] and Rubio [40] has shown that oral lysozyme can hydrolyze in the duodenum, producing antimicrobial peptides that are important for innate immunity. This process may also be responsible for the observed improvements in antioxidant status.

Elevated cortisol levels in the G2 group indicate a significant stress response to the infection. The decrease in total protein levels in the G2 group may be attributed to increased protein breakdown and altered metabolism due to the infection. Infection with *C. perfringens* and coccidia can trigger a significant stress response, leading to elevated cortisol levels [41]. Cortisol can have catabolic effects, leading to increased protein breakdown and potentially contributing to immunosuppression [42].

In contrast to findings in fish [43], the observed alterations in hematological indices in our study on broilers likely reflect the specific responses to *Eimeria* and *C. perfringens* infection and the subsequent therapeutic interventions.

The significant decrease in hemoglobin (Hb) and red blood cell count (RBCs) in the infected, untreated group (G2) suggests the development of anemia, potentially due to the parasitic and bacterial challenge impacting erythropoiesis or increasing erythrocyte destruction, as documented in poultry diseases [44].

The prophylactic lysozyme treatment (G3) showed a trend towards maintaining Hb and RBC levels closer to the control group (G1), suggesting a potential protective effect on red blood cell production in the face of subsequent infection. This could be linked to lysozyme's known immunomodulatory effects in poultry, potentially mitigating the severity of the infection and its impact on hematopoiesis [45].

The therapeutic lysozyme treatment (G4) also resulted in Hb and RBC values comparable to the control group, indicating a potential role in supporting recovery from the anemic effects of the infection. This might be due to lysozyme's ability to promote the clearance of pathogens and reduce inflammation, indirectly aiding the restoration of normal erythropoiesis [46].

Interestingly, the amoxicillin-treated group (G5) and the combination treatment group (G6) also showed Hb and RBC levels similar to the control, suggesting that both antibiotic and combination therapies were effective in addressing the underlying infection and its hematological consequences. However, G6 exhibited a significantly higher Hb level compared to the control, which could indicate a synergistic effect of lysozyme and amoxicillin on erythropoiesis or a more effective resolution of the disease-induced anemia [45].

Regarding the monocyte-macrophage system and immunoglobulins, lysozyme's promotion of their activity in poultry [10] could indirectly support erythropoiesis by enhancing pathogen clearance and reducing inflammatory cytokines that can suppress red blood cell production. The observed monocytosis in the infected group (G2) likely reflects the immune response to the pathogens, and the subsequent

modulation of monocyte levels in the treated groups could indicate the effectiveness of the interventions in controlling the infection and the associated immune activation.

Leucopenia in the G2 group is indicative of an immunosuppressed state, likely a consequence of the combined infections [47]. The observed heteropenia, lymphocytosis, and monocytosis in the G5 and G6 groups (supplemented with both lysozyme and antibiotics) may reflect the effects of antibiotic therapy on immune cell populations. Antibiotics can significantly alter the composition of the gut microbiota, which can in turn influence immune cell populations and their functions.

The current study examined the effects of lysozyme supplementation, both alone and in combination with amoxicillin, on gene expression profiles associated with oxidative stress, inflammation, and immunological response in broiler chickens infected with *Clostridium perfringens* and *Eimeria*.

The significant increase in *GSH* gene expression in the G6 group (lysozyme + amoxicillin) suggests an enhanced antioxidant defense mechanism. This finding aligns with previous studies demonstrating that lysozyme supplementation can modulate antioxidant systems in poultry [27]. The upregulation of *GSH*, a key antioxidant, likely contributes to mitigating oxidative stress induced by the infection [48].

Glutathione (GSH) is a crucial component of the endogenous antioxidant defense system against various xenobiotics. After hydrogen peroxide (H2O2) is produced by the action of superoxide dismutase (SOD), it is reduced to water by GSH and catalase [37,49]. GSH is abundantly present in the epithelial cells of the gastrointestinal tract (GIT), where it plays a vital role in the reduction of dietary peroxides [50].

The significant decrease in *IL-6* gene expression in the G6 group indicates a downregulation of the pro-inflammatory response. *IL-6* is a potent pro-inflammatory cytokine involved in various inflammatory processes [51]. The observed decrease in *IL-6* expression suggests that the combined treatment with lysozyme and amoxicillin may have effectively modulated the inflammatory response, limiting tissue damage.

The immunological response to the infection is likely the reason for the increased expression of *IL-1β* in all treatment groups compared to the infected control (G2). *IL-1β* is a crucial pro-inflammatory cytokine essential for the host's defense against infections [52].

The notable increases in *IL-10* gene expression in the G3 (prophylactic lysozyme) and G6 (lysozyme + amoxicillin) groups suggest an upregulation of anti-inflammatory responses. *IL-10*, an important anti-inflammatory cytokine, plays a key role in reducing excessive inflammation. The increased *IL-10* expression in these groups may contribute to a more balanced immune response, minimizing tissue damage while effectively controlling the infection [53].

The significant increase in *IgA* gene expression in all treatment groups indicates an enhanced mucosal immune response. *IgA* plays a crucial role in mucosal immunity, providing protection against pathogens at mucosal surfaces, such as the gut [54].

Interestingly, we also observed an increase in *IgY* gene expression in the G3 (prophylactic lysozyme) and G6 (lysozyme + amoxicillin) groups. While *IgA* is primarily associated with mucosal immunity, *IgY*, particularly in birds, plays a significant role in systemic immunity [55]. The observed increase in *IgY* gene expression suggests that the treatments may have stimulated a broader systemic immune response in addition to enhancing mucosal immunity.

This coordinated upregulation of both *IgA* and *IgY* suggests that the treatments may have effectively activated both mucosal and systemic immune responses, providing a multi-layered defense against *C. perfringens* infection. This broader immune response may contribute to improved overall health and disease resistance in the treated birds.

## Conclusion

This study provides compelling evidence for the multifaceted benefits of lysozyme supplementation in broiler chickens. By enhancing innate immunity, modulating the gut microbiota, and mitigating oxidative stress, lysozyme supplementation, particularly in combination with amoxicillin, significantly improved broiler performance, reduced the impact of *C. perfringens* infection, and strengthened the immune response.

The observed upregulation of both *IgA* and *IgY* gene expression suggests a coordinated activation of both mucosal and systemic immune responses, providing a robust defense against the infection.

These findings highlight the potential of lysozyme as a valuable feed additive for enhancing broiler health, optimizing production, and minimizing the reliance on antibiotics.

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*Declaration of Conflict of Interest*

None of the authors have any conflicting interests. *Ethical of approval*

This study was done according to the ethical guidelines of Assuit University, Egypt.

*Authors' contributions*

**A.S.E.-D.**: Writing - original draft, writing review and editing, conceptualization, formal analysis, investigation, methodology, resources, software, visualization, and validation. **A.M.M and M.A.G.:** Conceptualization, methodology, validation, resources**. H.M.M.:** Methodology. The final manuscript has been read and approved by all authors.

**TABLE 1. Primers sequences for SYBR green rt-PCR.**

| Target gene | Primers sequences | GenBank accession no. |
|---|---|---|
| *β*-actin | TTGTTGTGCAGGGTGTGATG<br>CTCTGTTGGCTTTGGGGTTC | X00182.1 |
| *IL-6* | TGTGCAAGAAGTTCACCGTG<br>ACTCGACGTTCTGCTTTTCG | AJ309540.1 |
| *IL-1β* | TCCTCCAGCCAGAAAGTGAG<br>GTCCAGGCGGTAGAAGATGA | NM_204524.2 |
| *IL-10* | AATGGCAGCTTAACGTTCGG<br>ACAACAGCACTGCCACAAAT | NM_001004414.4 |
| *IgA* | CAGGGCAATGAGTTCGTCTG<br>GGTCATCTCCTCGTTGTCCT | S40610.1 |
| *IgY* | CCAAGAACACTTCAACGGCA<br>GTAGATCAGAGGTGGGGTGG | LC706201.1 |
| *GSH* | GCATTTGTTGGGAGTGTGCT<br>GCATTTGTTGGGAGTGTGCT | BI067762.1 |

**TABLE 2.** Average Body Weight and Intestinal Lesion Scores of Broiler Chicks at 28 Days of Age

| Group | Average Body Weight (g) ± SD | Average Lesion Score |
|---|---|---|
| G1 (Control) | $1517 \pm 45^{a}$ | 0 |
| G2 (Infected Control) | $1330 \pm 38^{b}$ | 2.8 |
| G3 (Prophylactic Lysozyme) | $1490 \pm 42^{ab}$ | 1.6 |
| G4 (Therapeutic Lysozyme) | $1550 \pm 51^{a}$ | 1.2 |
| G5 (Therapeutic Amoxicillin) | $1500 \pm 48^{ab}$ | 1 |
| G6 (Lysozyme + Amoxicillin) | $1532 \pm 49^{a}$ | 0.8 |

a, b, and c: Significant differences ($p<0.05$) exist between mean values in the same row that have descriptive superscript letters that differ.

**TABLE 3. Mean Serum Levels (± Standard Error) of Malondialdehyde (MDA), Nitric Oxide (NO), Catalase (CAT), and Glutathione (GSH) in Study Groups**

| Parameter | G1 | G2 | G3 | G4 | G5 | G6 |
|---|---|---|---|---|---|---|
| **MDA (µmol/g protein)** | $10.78\pm1.28^{a}$ | $1267\pm56.3^{b}$ | $168.3\pm49.4^{c}$ | $10.4\pm3.4^{a}$ | $66.48\pm15.9^{a, c}$ | $16.11\pm1.13^{a}$ |
| **NO (nmol/g protein)** | $0.44\pm0.1^{a, c}$ | $2.39\pm0.7^{b}$ | $0.34\pm0.05^{a, c}$ | $0.25\pm0.02^{a, c}$ | $1.01\pm0.3^{a, b, c}$ | $0.51\pm0.1^{a, c}$ |
| **CAT (U/mg protein)** | $16.56\pm2.6^{a}$ | $1.23\pm0.16^{b}$ | $1.96\pm0.39^{b}$ | $27.00\pm2.5^{c}$ | $5.92\pm1.3^{b}$ | $6.53\pm0.93^{b}$ |
| **SOD (ng/mg protein)** | $2.21\pm0.44^{a}$ | $0.35\pm0.02^{b}$ | $1.08\pm0.05^{a, b}$ | $2.20\pm0.44^{a}$ | $1.62\pm0.25^{a, b}$ | $2.27\pm0.57^{a}$ |

**TABLE 4. Mean Serum Levels (± Standard Error) of Cortisol and Total Protein in Study Groups**

| Parameter | G1 | G2 | G3 | G4 | G5 | G6 |
|---|---|---|---|---|---|---|
| **Cortisol (nmol/l)** | 14.9 ± 1.6$^{a}$ | 57.6 ± 7.8$^{b}$ | 34.5 ± 1.1$^{c}$ | 20.4 ± 6.4$^{a}$ | 40.1 ± 5.9$^{b, c}$ | 26.9 ± 4.8$^{a, c}$ |
| **Total protein (g/ml)** | 8.77 ±0.8$^{a}$ | 4.91 ±0.6$^{b}$ | 7.25 ±1.2$^{a}$ | 7.33 ±1.1$^{a}$ | 5.10 ±0.3$^{b}$ | 6.35 ±1.1$^{a, b}$ |

**TABLE 5. Haematological Parameters in Study Groups (Mean ± SE)**

| Parameter | G1 | G2 | G3 | G4 | G5 | G6 |
|---|---|---|---|---|---|---|
| **Hb (g/dl)** | 12.49±0.4$^{a, c}$ | 10.84±0.1$^{b}$ | 11.99±0.2$^{a}$ | 12.02±0.2$^{a}$ | 12.25±0.2$^{a}$ | 13.33±0.3$^{c}$ |
| **PCV (%)** | 35.7±0.5$^{a}$ | 33.0±1.7$^{a}$ | 34.7±0.7$^{a}$ | 35.3±0.5$^{a}$ | 34.5±1.0$^{a}$ | 33.9±0.3$^{a}$ |
| **RBCs (x10$^{6}$/µl)** | 3.08±0.1$^{a}$ | 2.8±0.1$^{a}$ | 2.92±0.1$^{a}$ | 2.86±0.03$^{a}$ | 3.1±0.1$^{a}$ | 2.82±0.2$^{a}$ |
| **MCV (Fl)** | 40.78±3.4$^{a}$ | 38.79±0.9$^{b}$ | 41.11±0.9$^{a}$ | 41.43±0.9$^{a}$ | 38.56±0.4$^{b}$ | 47.69±3.3$^{c}$ |
| **MCHC (%)** | 35.87±0.9$^{a, b}$ | 32.99±1.9$^{a, b}$ | 34.58±1.3$^{a, b}$ | 33.65±1.3$^{a, b}$ | 34.74±0.5$^{a, b}$ | 39.31±1.2$^{a}$ |
| **WBCs (x10$^{3}$/µl)** | 33.22±0.2$^{a}$ | 30.42±0.1$^{b}$ | 35.34±0.6$^{a}$ | 33.41±0.9$^{a}$ | 33.12±0.2$^{a}$ | 35.59±0.5$^{a}$ |
| **Heterophils (%)** | 35.00±0.6$^{a}$ | 33.00±0.6$^{a}$ | 35.00±0.6$^{a}$ | 33.33±0.9$^{a}$ | 25.00±0.6$^{b}$ | 25.00±0.6$^{b}$ |
| **Lymphocyte (%)** | 50.00±1.2$^{a}$ | 48.00±1.1$^{a}$ | 48.33±0.8$^{a}$ | 49.33±0.9$^{a}$ | 55.33±1.4$^{b}$ | 61.67±0.3$^{b}$ |
| **Eosinophils (%)** | 4.33±0.3$^{a}$ | 4.00±0.5$^{a}$ | 4.33±0.3$^{a}$ | 4.00±0.1$^{a}$ | 5.00±0.6$^{a}$ | 6.67±0.3$^{b}$ |
| **Monocytes (%)** | 9.33±0.3$^{a}$ | 14.00±1.7$^{b}$ | 11.67±0.3$^{a, b}$ | 12.33±0.3$^{a, b}$ | 14.34±0.3$^{b}$ | 15.23±0.8$^{b}$ |

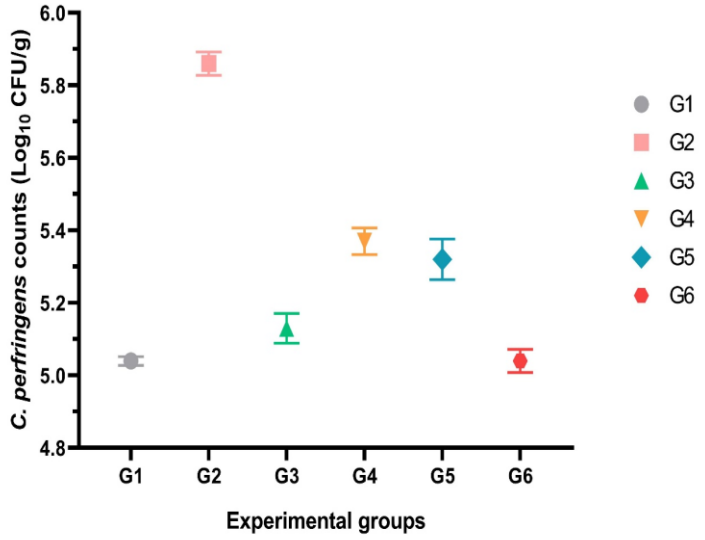


**Fig. 1. Effects of different treatments on *C. perfringens* counts before and after challenge.**

G1 (Control negative, CN): Uninfected and untreated birds, G2 (Control positive, CP): Birds infected with *Eimeria* and C. *perfringens*, G3: Birds offered prophylactic treatment with lysozyme (0.5 mL/L) in drinking water from day 9 to 13, G4: Birds offered therapeutic treatment with lysozyme (0.5 mL/L) in drinking water from day 20 to 24, G5: Birds offered therapeutic treatment with amoxicillin (20 mg/kg) in drinking water from day 20 to 24, and G6: Birds offered combination treatment with amoxicillin (20 mg/kg) and lysozyme (0.5 mL/L) in drinking water from day 20 to 24. Results are expressed as mean ± standard error of the mean (SEM).

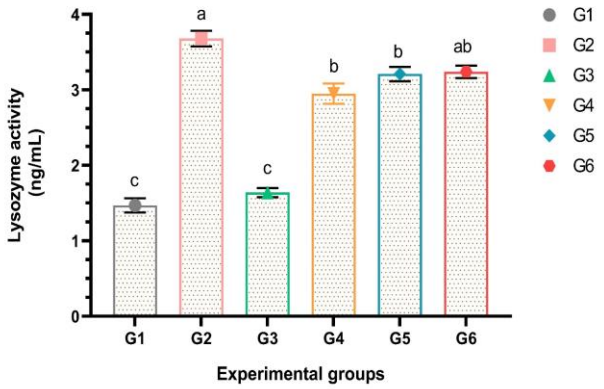


**Fig. 2. Effects of different treatments on lysozyme activity**.G1 (Control negative, CN): Uninfected and untreated birds, G2 (Control positive, CP): Birds infected with *Eimeria* and C. *perfringens*, G3: Birds offered prophylactic treatment with lysozyme (0.5 mL/L) in drinking water from day 9 to 13, G4: Birds offered therapeutic treatment with lysozyme (0.5 mL/L) in drinking water from day 20 to 24, G5: Birds offered therapeutic treatment with amoxicillin (20 mg/kg) in drinking water from day 20 to 24, and G6: Birds offered combination treatment with amoxicillin (20 mg/kg) and lysozyme (0.5 mL/L) in drinking water from day 20 to 24. Results are expressed as mean ± standard error of the mean (SEM). [a-c] Means with different superscript letters indicate significant differences at $p < 0.05$.

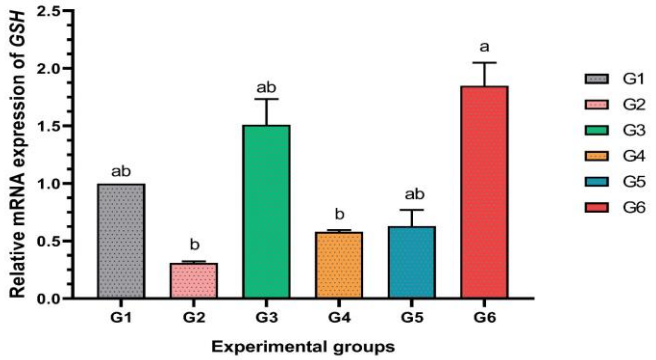


**Fig. 3. Relative mRNA expression levels of *GSH* gene.**
G1 (Control negative, CN): Uninfected and untreated birds, G2 (Control positive, CP): Birds infected with *Eimeria* and C. *perfringens*, G3: Birds offered prophylactic treatment with lysozyme (0.5 mL/L) in drinking water from day 9 to 13, G4: Birds offered therapeutic treatment with lysozyme (0.5 mL/L) in drinking water from day 20 to 24, G5: Birds offered therapeutic treatment with amoxicillin (20 mg/kg) in drinking water from day 20 to 24, and G6: Birds offered combination treatment with amoxicillin (20 mg/kg) and lysozyme (0.5 mL/L) in drinking water from day 20 to 24. Results are expressed as mean ± standard error of the mean (SEM). [a-b] Means with different superscript letters indicate significant differences at $p < 0.05$.

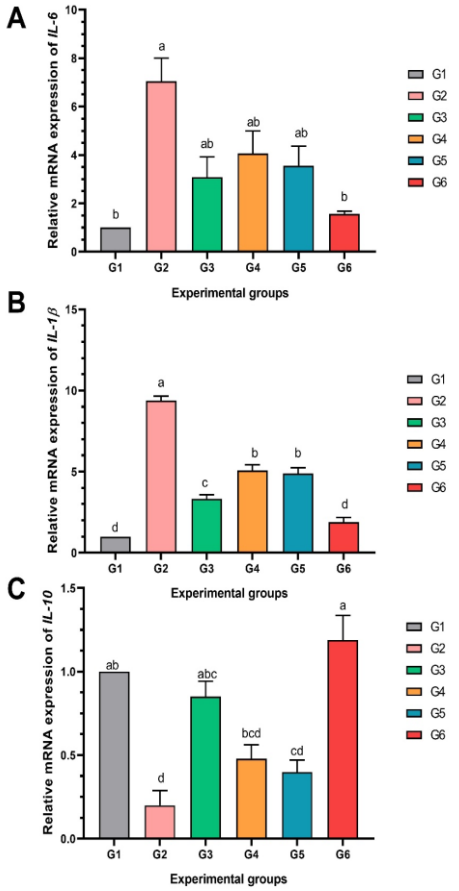


**Fig. 4. Relative mRNA expression levels of *IL-6* (A), *IL-1β* (B), and *IL-10* (C) genes.**

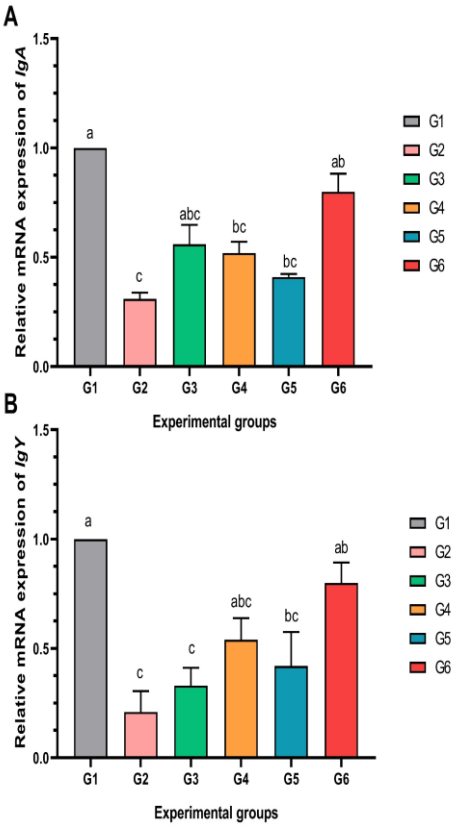


**Fig. 5. Relative mRNA expression levels of *IgA*(A), and *IgY* (B) genes.**

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# تقييم تأثير إضافة الليزوزيم على حالة مضادات الأكسدة، والتعبير الجيني المناعي، وتخفيف التهاب الأمعاء التنكرزي في دجاج التسمين

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**الملخص**

هدفت هذه الدراسة إلى تقييم فعالية إضافة إنزيم الليزوزيم، بمفرده وبالاشتراك مع الأموكسيسيلين، في تخفيف الآثار الضارة للعدوى المشتركة بين الكلوستريديوم بيرفرنجنز والإيميريا في دجاج التسمين. تم تقسيم مائة وعشرين كتكوتًا عمر يوم واحد عشوائيًا إلى ست مجموعات: مجموعة– ضابطة غير مصابة، مجموعة ضابطة مصابة، مجموعة ليزوزيم وقائية، مجموعة ليزوزيم علاجية، مجموعة أموكسيسيلين علاجية، ومجموعة ليزوزيم + أموكسيسيلين علاجية. تم تحفيز الإصابة في الطيور بـالكلوستريديوم بيرفرنجنز والإيميريا. أدت إضافة الليزوزيم إلى تحسين معنوي في أداء النمو، وتقليل الإجهاد التأكسدي، وتطبيع مستويات الكورتيزول، وتعزيز المعلمات الدموية (بما في ذلك عدد الخلايا الحمراء و الكريات البيضاء). لوحظ ارتفاع معنوي في نشاط الليزوزيم في مصل الدم في المجموعات المعالجة بالليزوزيم. أظهرت المجموعة السادسة (الليزوزيم + الأموكسيسيلين) أكثر التحسينات وضوحًا في جميع القياسات التي تم تقييمها. أظهرت هذه المجموعة أدنى عدد الكلوستريديوم بيرفرنجنز ، وانخفاض في علامات الإجهاد التأكسدي ، وزيادة في مستويات مضادات الأكسدة، وتطبيع مستويات الكورتيزول والبروتين. كشف تحليل البي سي ار الكمي عن زيادة تعبير جين GSH وانخفاض تعبير جين IL-6 في المجموعة السادسة. أظهرت جميع مجموعات العلاج زيادة في تعبير جيني ال IL-1β و IgA، بينما تم تنظيم تعبير جين IgY في المجموعتين الثالثة (الليزوزيم الوقائي) والسادسة. تُظهر هذه النتائج أن إضافة الليزوزيم، وخاصةً بالاشتراك مع الأموكسيسيلين، يخفف بشكل فعال الآثار الضارة للعدوى المشتركة بين بين الكلوستريديوم بيرفرنجنز والإيميريا في دجاج التسمين. يمكن أن تُعزى التحسينات الملحوظة إلى تعزيز الاستجابات المناعية، وتحسين حالة مضادات الأكسدة، وتطبيع المعلمات الفسيولوجية، وتقليل العبء البكتيري، مما يُبرز إمكانات الليزوزيم كأداة قيّمة لتعزيز صحة الدجاج وتحسين الإنتاج.

**الكلمات الدالة:** كلوستريديوم بيرفرنجنز، عدوى مشتركة، مُضافات علفية، الميكروبات المعويه، المناعة المخاطية، الإجهاد التأكسدي، صحة الدواجن.