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Unveiling the Molecular Crosstalk between Heat Stress, Vaccination, and *E. coli* Infection in Broilers

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Abstract

Escherichia coli infections pose a major threat to poultry health, with biofilm formation significantly contributing to disease. This study uniquely investigated the complex interactions between vaccination, an *E. coli* challenge, and heat stress on broilers, addressing a research gap that typically examines these factors in isolation. Vaccination proved highly effective, substantially reducing mortality and clinical signs while significantly lowering cecal *E. coli* load in challenged birds. At a molecular level, vaccination downregulated all four key biofilm genes and enhanced antioxidant enzyme expression. Our most notable finding was a statistically significant, synergistic inflammatory response in the triple-stressed group (vaccinated, infected, and heat-stressed), which exhibited the highest pro-inflammatory cytokine expression. These results underscore the importance of considering multiple stressors in poultry health. The findings directly inform poultry management by supporting vaccination as a comprehensive strategy to boost broiler resilience, particularly in regions prone to heat stress. Furthermore, this study provides a molecular roadmap for developing next-generation vaccines that not only elicit an immune response but also prime a bird's physiological systems to better cope with combined environmental and pathogenic challenges.

Keywords: Biofilm gene expression; Immune modulation; Poultry health; Stress response; Vaccination.

Introduction

The poultry industry faces significant economic losses due to infectious diseases caused by bacterial pathogens like *Escherichia coli*. These infections lead to increased mortality, reduced growth rates, and decreased egg production [1–3]. A key factor in the persistence and severity of *E. coli* infections is biofilm formation. Biofilm-embedded bacteria are more resistant to antibiotics, disinfectants, and the host's immune system, making these infections incredibly difficult to eradicate and often leading to chronic outbreaks [4–6].

Compounding this challenge is heat stress, a common environmental factor that can worsen the effects of infectious disease [7, 8]. High temperatures can damage the intestinal barrier, allowing bacteria to enter the bloodstream more easily. Heat stress also suppresses the immune

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response, making birds more vulnerable to infection and creating an ideal environment for biofilm-forming *E. coli* to flourish [9].

Vaccination is a cornerstone of disease prevention in poultry. However, the effectiveness of vaccines can be significantly compromised under conditions of heat stress, as high temperatures can impair the immune response, reduce antibody production, and hinder the development of protective immunity [10, 11].

While previous studies have investigated the individual effects of *E. coli* infection, heat stress, and vaccination, there is a critical gap in understanding how these factors interact. Our study is the first to systematically examine the complex, combined effects of these three stressors on broilers. We move beyond simply observing outcomes by investigating the underlying molecular mechanisms. Specifically, we analyzed changes in biofilm gene expression, key pro-inflammatory cytokine responses, and antioxidant enzyme systems in groups of birds subjected to vaccination, *E. coli* challenge, and heat stress simultaneously.

The findings from this research will provide a new, multi-level understanding of how a bird's body responds to combined environmental and pathogenic threats. This work offers a crucial molecular roadmap for developing next-generation vaccines and management strategies designed to build resilience in poultry, particularly in regions prone to heat stress.

Material and Methods

Ethics approval

All methods were performed in accordance with the relevant guidelines and regulations in accordance with the ARRIVE guidelines (PLoS Biol 8(6), e1000412, 2010) and approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine, Assiut University, Egypt (Approval number: 06/2024/0213).

Sample Collection and Bacterial Isolation

A total of 100 broiler chickens, aged 32-45 days, exhibiting symptoms of diarrhea and colisepticemia (pericarditis and airsaculitis) were randomly collected from 17 farms in Sharkia Governorate, Egypt, between January and August 2024. Prior to sample collection, verbal consent was obtained from each of the 17 farm owners, explicitly permitting the collection and use of samples from the affected birds for this research study. Following euthanasia, internal organs (lungs, liver, and heart) were aseptically collected from each bird and pooled. Samples were immediately transported to the Bacteriology Laboratory for further analysis. All collected samples were subjected to ***E. coli*** isolation and identification according to Quinn et al. [15]. Confirmed *E. coli* isolates were serotyped using known antisera from Sifin, following the protocol of Lee et al. [16].

Biofilm Formation Assays

a. Congo Red Agar Method

Biofilm formation was qualitatively assessed using Congo Red Agar (CRA) as described by Freeman et al. [17]. Briefly, bacterial isolates were inoculated onto CRA plates and incubated at 37°C for 24-48 hours. Colonies with a black, dry, and wrinkled appearance were considered strong biofilm producers, while red, smooth colonies were considered non-biofilm producers. Intermediate phenotypes were also observed.

b. Quantitative Biofilm Assay

Quantitative biofilm formation was determined using a microtiter plate assay as described by Stepanović et al. [18, 19]. Briefly, bacterial cultures were grown overnight in TSB, diluted to an OD_{600} of 0.5, and inoculated into 96-well microtiter plates. After incubation at 37°C for 24 hours, the wells were washed with sterile phosphate-buffered saline (PBS) to remove non-adherent bacteria. The remaining biofilm was stained with 0.1% crystal violet for 15 minutes, washed again with PBS, and then dissolved in 33% acetic acid. The absorbance at 570 nm was measured using a microplate reader to quantify biofilm biomass. The optical density (OD) of each well was compared to the OD of a negative control (sterile TSB).

Biofilm categorization was based on the following standardized optical density (OD) cutoff values, where ODc represents the mean optical density of the negative control: a sample was classified as a non-biofilm producer if its OD was less than or equal to ODc, a weak biofilm producer if its OD was between ODc and 2x ODc, a moderate biofilm producer if its OD was between 2x ODc and 4x ODc, and a strong biofilm producer if its OD was greater than 4x ODc. This methodology, adapted from Stepanović et al., ensures a robust and consistent categorization of our biofilm results.

Identification and Characterization of the Strong Biofilm-Producing E. coli *Challenge Isolate*

To establish a robust experimental model, the *E. coli* isolate exhibiting the strongest biofilm-forming ability was selected as the challenge strain. Genomic DNA was extracted from the isolate using a QIAamp DNA Mini Kit (Qiagen, Germany). The 16S rRNA gene was then amplified using universal primers 27F and 1492R [20]. The PCR product was visualized on

an agarose gel stained with ethidium bromide (0.5 g/mL).

Subsequently, Sanger sequencing was performed on an Applied Biosystems 3130 automated DNA Sequencer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer/Applied Biosystems, Foster City, CA). The obtained sequence was compared to sequences in the GenBank database using BLAST to confirm the isolate's identity.

Experimental Design

A total of 200 one-day-old Arbor Acres broiler chicks were randomly allocated to five experimental groups:

Group 1 (C-ve): Non-challenged, non-vaccinated

Group 2 (C+ve): Challenged with *E. coli*, non-vaccinated

Group 3 (V+I): Vaccinated and challenged

Group 4 (V+I+H): Vaccinated, challenged, and subjected to heat stress (elevated temperature above 35°C)

Group 5 **(V):** Vaccinated only

Justification for Timing of Stressors

The heat stress was applied from day 1 to 21 of age, and the *E. coli* challenge was administered on day 21. This design was specifically chosen to simulate a common real-world scenario where an early-life heat wave can chronically stress broiler chicks, potentially compromising their immune system's ability to respond to a subsequent infection after the heat stress has subsided. This approach allowed us to isolate and evaluate the long-term effects of early heat stress on vaccine efficacy and the bird's overall resilience to *E. coli* infection.

Vaccination

Birds in the vaccinated groups (Groups 3, 4, and 5) were administered a single intramuscular dose of Poulvac® *E. coli* vaccine (Zoetis) at one week of age. The vaccine, containing live *Escherichia coli* serotype O78 strain EC34195, was reconstituted according to the manufacturer's instructions [21] and administered at a dose of 10^9 CFU/bird.

E. coli Challenge

Groups 2, 3, and 4 were intratracheally challenged with 1 mL of a suspension containing $6x10^8$ colony-forming units (CFU) of the isolated *E. coli* at 21 days of age. The challenge dose was determined based on previous studies [22].

Heat Stress

Group 4 was subjected to a chronic heat stress protocol to simulate a severe heat wave. The ambient temperature was maintained at a constant 35 ± 1°C for 8 hours per day, from 09:00 to 17:00, with the remaining 16 hours at a thermoneutral temperature of 25 ± 1°C. This heat stress was applied daily from day 1 to 21 of age. The relative humidity within the housing environment was kept at approximately 60%. It is important to note that the heat stress was ceased prior to the *E. coli* challenge on day 21. This approach models the long-term physiological impact of an early-life heat event on vaccine effectiveness, allowing us to evaluate vaccine efficacy in birds whose systems were already stressed.

Clinical Observations

Daily monitoring of clinical signs (diarrhea, respiratory distress, mortality) was conducted.

Euthanasia Process

Birds were subjected to 45% carbon dioxide gas for euthanasia, resulting in rapid loss of consciousness [23].

Necropsy

Gross pathological lesions were evaluated in all birds at the end of the experiment. Lesions in the air sacs, heart, and pericardium, and liver were scored as follows: normal (0), mild (1), moderate (2), and severe (3), according to Peighambari et al. [24].

Quantification of the Challenge E. coli Strain

Cecal swabs were collected from all birds at the end of the experiment, serially diluted, and plated on MacConkey agar containing cefotaxime (2 µg/mL). This selective medium was used to specifically quantify the bacterial load of the cefotaxime-resistant challenge *E. coli* strain, differentiating it from the general commensal bacterial population [25, 26].

Tissue Collection

Liver, cecum, and spleen tissues were aseptically collected from all birds at the end of the experiment for RNA extraction and subsequent gene expression analysis.

Pathogen Monitoring

To ensure the absence of other pathogens, random cloacal swabs were collected every 3 days and subjected to PCR and culture-based diagnostic methods.

RNA Extraction and Gene Expression Analysis

Total RNA was extracted from tissue samples using the QIAamp RNA Mini Kit (Qiagen, Germany). One-step RT-qPCR was performed using specific primers (Table 1) and a HERA SYBR® Green One-Step RT-qPCR Master Mix (Willowfort, UK) on a StepOne Real-Time PCR System (Applied

Biosystems) established in the Biotechnology Unit, Animal Health Research Institute, Zagazig Branch, Egypt. Each 20 µL reaction mixture contained 10 µL of 2X One-Step RT-qPCR Master Mix, 0.5 µL of each primer (20 pmol/µL), 3 µL of RNA template, and 6.5 µL of nuclease-free water. The PCR cycling conditions were as follows: initial denaturation at 94°C for 15 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 10 min.

Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [27, 28]**.** The expression of target genes (including inflammatory cytokines, antioxidant, heat shock, and heat stress proteins) was normalized to the housekeeping gene β-actin. For bacterial biofilm genes, expression was normalized to the housekeeping gene 16S rRNA.

Justification for Housekeeping Genes

β-actin was selected as the housekeeping gene for host gene expression due to its well-established role as a reliable reference gene in poultry research. It is involved in cell motility and structure and is widely recognized for its stable expression across various tissues and under many experimental conditions [29].

16S rRNA was chosen for bacterial gene expression normalization because of its stable and high-level expression across different growth conditions and its essential role in bacterial ribosome function. This makes it an ideal reference gene for quantifying the expression of other bacterial genes [30].

Statistical Analysis

Statistical analysis was performed using SPSS 27.0 software (IBM Corp., Chicago, United States). Prior to analysis, the normality of all continuous data was assessed using the Shapiro-Wilk test. For data that met the assumption of normality, a one-way analysis of variance (ANOVA) was employed to determine significant differences among the means of the evaluated parameters. Tukey's HSD test was used for pairwise comparisons. For data that did not follow a normal distribution, the non-parametric Kruskal-Wallis test was used, followed by Dunn's test for pairwise comparisons. Mortality rates, as time-to-event data, were analyzed using Kaplan-Meier survival analysis, with the Log-Rank test used to compare the survival curves between the different experimental groups. A significance level of $p < 0.05$ was used for all tests. All figures were generated using GraphPad Prism (United States).

Results

Isolation and Serotyping of E. coli

E. coli was isolated from 44 of 100 (44%) examined broiler chickens. Standard microbiological techniques were used to identify the isolates. Serotyping revealed three predominant serotypes: O78 (50%), O1 (29.5%), and O2 (20.5%).

Biofilm Formation and Sequencing Data

Of the 44 *E. coli* isolates, 10 (22.7%) exhibited strong biofilm-forming ability, as indicated by black colony formation on Congo Red Agar plates. The remaining 34 isolates were classified as weak or non-biofilm producers based on their red colony morphology. Quantitative analysis using the microtiter plate assay further confirmed these results, with all isolates demonstrating some level of biofilm formation. However, only one isolate, serotype O78, was identified as a strong biofilm producer. The majority of isolates were classified as moderate biofilm producers (Figure 1).

To further characterize this potent biofilm-forming *E. coli* O78 isolate and use it as a challenge strain, 16S rRNA gene sequencing was performed. The obtained sequence was deposited in GenBank under accession number PQ764122.

In vivo Observations

Clinical Signs

Following *E. coli* challenge, birds in the positive control group (Group 2; C+ve) exhibited prominent clinical signs, including decreased appetite, depression, ruffled feathers, nasal discharge, coughing, sneezing, and brown-colored diarrhea. Birds in the vaccinated and challenged group (Group 3; V+I) displayed less severe clinical signs compared to the positive control, particularly with respect to respiratory symptoms. The most severe clinical manifestations were observed in the group subjected to both vaccination, challenge, and heat stress (Group 4; V+I+H), with pronounced respiratory distress, suggesting a synergistic effect of the combined stressors. No clinical signs were observed in the control group (Group 1; C-ve) or the vaccinated-only group (Group 5; V).

Mortality rates

Mortality was highest in the positive control group (22.5%), followed by the heat-stressed group (12.5%). No mortality was observed in the control and vaccinated-only groups (Table 2).

Gross Necropsy Findings

The positive control group showed the most severe lesions, including severe air sacculitis, marked pericarditis, and marked perihepatitis. The vaccinated

and challenged group exhibited mild air sacculitis and pericarditis, while the heat-stressed group showed severe air sacculitis and pericarditis with minor liver lesions (Table 3).

Effect on Antioxidant Enzyme Gene Expression

The expression of antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), was significantly decreased in the positive control group, indicating oxidative stress. In contrast, all treatment groups, including the vaccinated groups, showed significantly increased expression of SOD and CAT, suggesting a protective response against oxidative stress (Figure 3).

Effect of Vaccination, Challenge, and Heat Stress on Cytokine Gene Expression

The mRNA expression levels of pro-inflammatory cytokines (*IL-1β, IL-6*, *TNF-α,* and *IFN-γ*) and the toll-like receptor (*TLR-4*) were significantly upregulated in the positive control group and all treatment groups compared to the negative control group. This suggests that both *E. coli* infection and heat stress can induce a robust inflammatory response.

Interestingly, the V+I+H group exhibited the highest levels of pro-inflammatory cytokine expression, indicating a synergistic effect of vaccination, challenge, and heat stress. In contrast, the V group, which received only the vaccine, showed no significant difference in pro-inflammatory cytokine expression compared to the negative control, suggesting that vaccination alone may not induce a strong inflammatory response (Fig.2)**.**

Effect on Stress Response Gene Expression

The expression of stress-responsive transcription factors, such as p38 MAPK, NF-κB, HSF1, and HSF3, was significantly downregulated in the V+I+H and V groups compared to the positive control (Fig.4)**.** This suggests that vaccination may have a protective effect against the adverse effects of heat stress.

The expression of heat shock proteins (HSP-90 and HSP-70) was significantly upregulated in the H+I+V group compared to the negative control, indicating a strong heat stress response. However, the V group showed lower expression of HSPs compared to the positive control, suggesting that vaccination may have a mitigating effect on heat stress-induced protein damage (Fig 5).

Bacterial Load in the Cecum

The cecal bacterial load of *E. coli* was significantly higher in the positive control and all treatment groups compared to the negative control. However, the V group showed significantly lower bacterial load compared to the other treatment groups, indicating that vaccination can reduce bacterial colonization in the gut (Fig. 6).

These results suggest that vaccination can provide some protection against *E. coli* infection, even under heat stress conditions. However, the combined effects of heat stress and infection can overwhelm the immune response and lead to increased bacterial load and inflammation.

Modulatory Effect of Vaccination, Challenge, and Heat Stress on Biofilm Gene Expression

The results investigated the impact of vaccination, *E. coli* challenge, and heat stress on the expression of biofilm-related genes (*fimA, ompA, csgA,* and *fimH*) in broiler chicks. Compared to the challenged group, vaccination significantly downregulated the expression of all four biofilm genes. The combination of vaccination and challenge resulted in further suppression of biofilm gene expression compared to the challenged group alone. Heat stress in vaccinated and challenged birds appeared to have a variable effect on biofilm gene expression, with some genes showing further downregulation. These findings suggest that vaccination provides some level of protection against *E. coli*-induced biofilm formation, and this effect may be influenced by subsequent heat stress (Fig.7).

Discussion

The isolation of *E. coli* from 44% of the examined broiler chickens underscores the prevalence of this pathogen in poultry. Serotyping revealed a predominance of O78, O1, and O2 serogroups, which are commonly associated with avian pathogenic *E. coli* (APEC) infections [31–33]. These serotypes possess specific virulence factors that facilitate colonization of the avian host and enhance their ability to form biofilms, which contribute to increased resistance to antimicrobial agents and host immune defenses [32, 34, 35]. To mitigate the impact of *E. coli* infections, it is crucial to implement robust biosecurity measures, optimize management practices, and consider vaccination strategies.

The prevalence of biofilm-forming *E. coli* strains, particularly serotype O78, highlights the significant role of biofilms in the persistence and virulence of these pathogens in poultry. Heat stress can induce physiological stress in birds, compromising their immune response and creating a favorable environment for bacterial colonization and biofilm formation [36, 37].

The virulence of *E. coli* strains is influenced by the presence of various virulence factors, including adhesins and extracellular matrix components, which

facilitate bacterial colonization and biofilm formation [38, 39]. These factors contribute to the severity of infection by enabling the bacteria to evade host defenses and establish persistent infections. Understanding the molecular mechanisms underlying biofilm formation and the interplay between *E. coli*, the host immune system, and environmental stressors like heat stress is crucial for developing effective strategies to control these infections.

Consistent with previous studies [40–42], the positive control group (Group 2; C+ve) exhibited the most severe clinical signs, including decreased appetite, depression, ruffled feathers, nasal discharge, coughing, sneezing, and brown-colored diarrhea. Vaccination in group 3 (V+I) mitigated the severity of clinical signs, aligning with the expected protective effect of vaccination against *E. coli* infections [43–45]. However, the combination of challenge and heat stress in group 4 (V+I+H) exacerbated clinical signs, with pronounced respiratory distress, suggesting that heat stress can compromise the effectiveness of the vaccine and increase disease severity, as observed in previous studies on the impact of heat stress on poultry immune function [46, 47]. No clinical signs were observed in the control group (Group 1; C-ve) or the vaccinated-only group (Group 5; V).

Mortality rates followed a similar pattern. The highest mortality rate was observed in the positive control group (22.5%), followed by the heat-stressed group (12.5%). No mortality occurred in the control and vaccinated-only groups. These findings align with previous studies demonstrating increased mortality rates in *E. coli*-challenged birds [48, 49] and the detrimental impact of heat stress on disease outcome in poultry [12, 50].

Gross necropsy findings mirrored the clinical observations. The positive control group exhibited the most severe lesions, including severe air sacculitis, pericarditis, and perihepatitis, consistent with previous reports on the pathological changes associated with *E. coli* infections in poultry [51, 52]. The vaccinated and challenged group showed milder lesions, indicating a degree of protection afforded by vaccination. Notably, the heat-stressed group exhibited severe lesions similar to the positive control, suggesting that heat stress can exacerbate the severity of *E. coli*-induced lesions, despite vaccination, as previously reported in the literature [53, 54].

The findings of this study provide valuable insights into the complex interplay between heat stress, vaccination, and *E. coli* infection in broiler chickens. We observed a significant upregulation of pro-inflammatory cytokines (*IL-1β, IL-6*, *TNF-α,* and *IFN-γ*) and *TLR*-4 in infected and heat-stressed birds, indicating a robust inflammatory response. These pro-inflammatory cytokines play a crucial role in initiating and amplifying the immune response to infection. *TLR*-4, a key pattern recognition receptor, recognizes pathogen-associated molecular patterns (PAMPs) from *E. coli* and triggers the activation of immune cells [55, 56].

However, the vaccinated groups exhibited a more controlled inflammatory response, suggesting that vaccination can modulate the host's immune response. The vaccine likely induces the production of specific antibodies that can neutralize *E. coli* and prevent bacterial colonization of the intestinal tract [57]. Additionally, vaccination may enhance the innate immune response by priming immune cells to respond more effectively to infection [58, 59].

The increased expression of antioxidant enzymes, such as superoxide dismutase (*SOD*) and catalase (*CAT*), observed in all treatment groups, including the vaccinated ones, indicates a protective response against the oxidative stress induced by heat stress and bacterial infection [60]. *SOD* catalyzes the dismutation of superoxide radicals into hydrogen peroxide [61], while *CAT* further reduces hydrogen peroxide into water and oxygen. By effectively scavenging reactive oxygen species (ROS), these enzymes help to mitigate oxidative damage to cellular components, including DNA, proteins, and lipids [61–63]. This protective response is crucial for maintaining cellular integrity and function, especially under stress conditions.

The upregulation of antioxidant enzymes in vaccinated birds suggests that vaccination may stimulate a broader immune response, including the activation of antioxidant defense mechanisms [59]. This could contribute to the improved health and performance of vaccinated birds, particularly when exposed to additional stressors such as heat stress.

The downregulation of stress-responsive transcription factors (*p38 MAPK, NF-κB, HSF1*, and *HSF3*) in vaccinated groups suggests that vaccination may prime the immune system to better manage cellular stress [64–66]. This could potentially reduce the severity of heat stress-induced damage by mitigating inflammatory responses and cellular damage.

Furthermore, the upregulation of heat shock proteins (*HSP-90* and *HSP-70*) in the heat-stressed vaccinated group indicates a more robust cellular stress response. These proteins play a crucial role in protein folding, refolding, and degradation, protecting cells from damage caused by heat stress [67, 68]. The enhanced expression of HSPs in vaccinated birds suggests that vaccination may

enhance the cellular stress response, providing additional protection against heat stress.

The increased bacterial load in the ceca of infected birds highlights the importance of effective immune responses in controlling bacterial colonization. The reduced bacterial load in the vaccinated group suggests that vaccination can enhance gut barrier function and limit bacterial invasion [69, 70].

Biofilms provide a significant advantage for bacteria, shielding them from environmental stressors, including heat stress [71]. Within the biofilm matrix, bacteria exhibit increased resistance to antimicrobial agents, desiccation, and host immune responses [72–74]. This enhanced resistance can exacerbate the negative impacts of heat stress on the host by promoting bacterial persistence and hindering effective immune clearance[75, 76].

Key genes involved in biofilm formation, such as *fimA* and *fimH* (encoding type 1 fimbriae for adherence, [77, 78], *ompA* (involved in cell envelope structure and function, [79], and *csgA* (a major component of the extracellular matrix, [80, 81], were significantly downregulated in vaccinated birds. This finding presents a crucial question regarding the underlying mechanism: does vaccination indirectly reduce biofilm formation by simply lowering the overall bacterial load, or does it directly modulate the expression of these genes, potentially influencing the pathogen's virulence strategy? Our observation of a significantly lower cecal *E. coli* load in vaccinated birds strongly supports the former hypothesis, where effective immune clearance reduces the bacterial population available to form biofilms. However, we cannot rule out a direct modulatory effect, where the vaccine-induced immune response, such as specific antibodies or other host-derived molecules, might interfere with bacterial quorum sensing or other signaling pathways that govern biofilm gene expression. While our study demonstrates a clear correlation between vaccination and reduced biofilm gene expression, further research would be needed to definitively disentangle these two potential mechanisms. This finding suggests that vaccination effectively modulates host immune responses, leading to reduced bacterial colonization and, consequently, diminished biofilm formation [82–84]. While heat stress can compromise host defenses and potentially enhance biofilm formation, our results indicate that vaccination may offer some level of protection against these detrimental effects. These findings have crucial implications for developing effective strategies to control *E. coli* infections and mitigate the negative impacts of heat stress on poultry production.

Conclusion

This study provides valuable insights into the complex interplay between vaccination, *E. coli* infection, and heat stress in broiler chickens. Our findings demonstrate that vaccination can significantly reduce bacterial colonization by downregulating the expression of biofilm-related genes, a crucial step in *E. coli* pathogenesis. However, the combined effects of *E. coli* challenge and heat stress can significantly disrupt host homeostasis, leading to increased inflammation, oxidative stress, and potentially compromising immune function. While vaccination offers some protection against these combined stressors, further research is warranted to investigate novel vaccination strategies, explore the use of immune-stimulants, and develop targeted interventions to mitigate the adverse effects of heat stress on poultry health and productivity. These findings emphasize the need for a multi-pronged approach to combating bacterial infections in poultry, considering the multifaceted nature of environmental and immunological challenges.

Funding statement

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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

M.A.G.: Conceptualization, Methodology, Validation, Resources. A.M.K.: Conceptualization and Methodology. R.I.H. and S.S.E.L.: Methodology. A.S.E.-D.: Conceptualization, Formal Analysis, Investigation, Methodology (Molecular and Bacteriology), Resources, Software, Visualization, Validation, Writing – Original Draft, Writing – Review & Editing. All authors have read and approved the final manuscript.

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

Target gene	Primers sequences	Reference
Host primers:		
***β*-actin**	CAACACAGTGCTGTCTGGTGG ATCGTACTCCTGCTTGCTGAT	[74]
IL-6	CCCGCTTCTGACTGTGTTT GCCGGTTTTGAAGTTAATCTTT	[75]
IL-1β	TGCTGGTTTCCATCTCGTATGTAC CCCAGAGCGGCTATTCCA	[75]
TNF-α	CCCCTACCCTGTCCCACAA ACTGCGGAGGGTTCATTCC	[75]
IFN-γ	AACAACCTTCCTGATGGCGTGA GCTTTGCGCTGGATTCTCAAGT	[76]
TLR-4	ACCCATTGTCACCAACATCATC TGCCTCAGCAAGGTCTTATTCA	[77]
HSP-70	CGGGCAAGTTTGAC CTAA TTGGCTCCCACCCTATCTCT	[78]
HSP-90	TCCTGTCCTGGCTTTAGTTT AGGTGGCATCTCCTCGGT	[78]
HSF1	TGTGGCTGATTCTTGGCTTT GAGGGAGACAGAGGGGTTTC	[79]
HSF3	CGGAAGATGGAAATGGAGAG TCAGGAAGCAGGAGAGGAGA	[79]
SOD	CACTGCATCATTGGCCGTACCA GCTTGCACACGGAAGAGCAAGT	[80]
CAT	TGGCGGTAGGAGTCTGGTCT GTCCCGTCCGTCAGCCATTT	[80]
NF-κB	TACTGATTGCTGCTGGAGTTGATGTC TTGTGCCATCGTATGTAGTGCTGTC	[76]
p38MAPK	AGGAGCTCAACAAGACGGTG AGCCGTAGTTCTCGGTAGGT	[81]
Bacterial primers:		
fimH	GTGCCAATTCCTCTTACCGTT TGGAATAATCGTACCGTTGCG	[82]
csgA	TGGTAACAGCGCCACTCTTG GACGGTGGAATTAGATGCAGTC	[83]
ompA	TCCAGAGCAGCCTGACCTTC GCTGAGCCTGGGTGTTTCCT	[84]
fimA	TGTCCCTCAGTTCTACAGCG TCCTAACTGAACGGTTTGATC	[85]

TABLE 2. Mortality rates by groups.

Groups	Mortality (n/40)	Mortality Rate (%)
C-ve	0/40[b]	0%[b]
C+ve	9/40[a]	22.5%[a]
V+I	1/40[b]	2.5%[b]
V+I+H	5/40[a]	12.5%[a]
V	0/40[b]	0%[b]

Values with different superscript letters in the same column are significantly different ($p < 0.05$) based on the Chi-square test with post-hoc pairwise comparisons.

TABLE 3. Gross Necropsy Findings by groups

Organs	C-ve	C+ve	V+I	V+I+H	V
Air Sacs	Score 0 (Normal)[b]	Score 3 (Severe)[a]	Score 1 (Mild)[c]	Score 3 (Severe)[a]	Score 0 (Normal)[b]
Heart and Pericardium	Score 0 (Normal)[b]	Score 2 (Marked)[a]	Score 1 (Mild)[c]	Score 2 (Marked)[a]	Score 0 (Normal)[b]
Liver	Score 0 (Normal)[c]	Score 2 (Marked)[a]	Score 0 (Normal)[c]	Score 1 (Minor)[b]	Score 0 (Normal)[c]

Score 3 (Severe) in air sacs indicates air sacculitis; thickening and cloudiness with mucopurulent or fibrinopurulent exudate; score 2 (Marked) in heart and pericardium indicates marked pericarditis, and score 2 (Marked) in liver indicates marked perihepatitis. Values with different superscript letters in the same row are significantly different ($p < 0.05$) based on the Kruskal-Wallis test with Dunn's post-hoc pairwise comparisons.

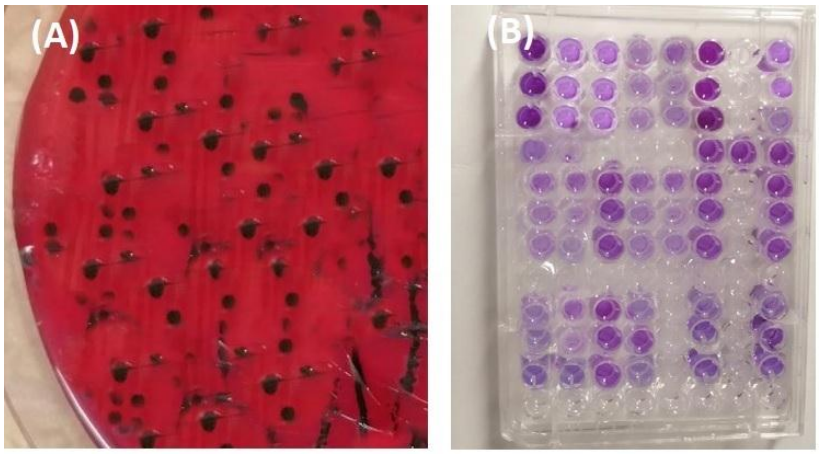

Fig. 1. Biofilm formation on Congo Red Agar (A) and Microtiter plate (B).

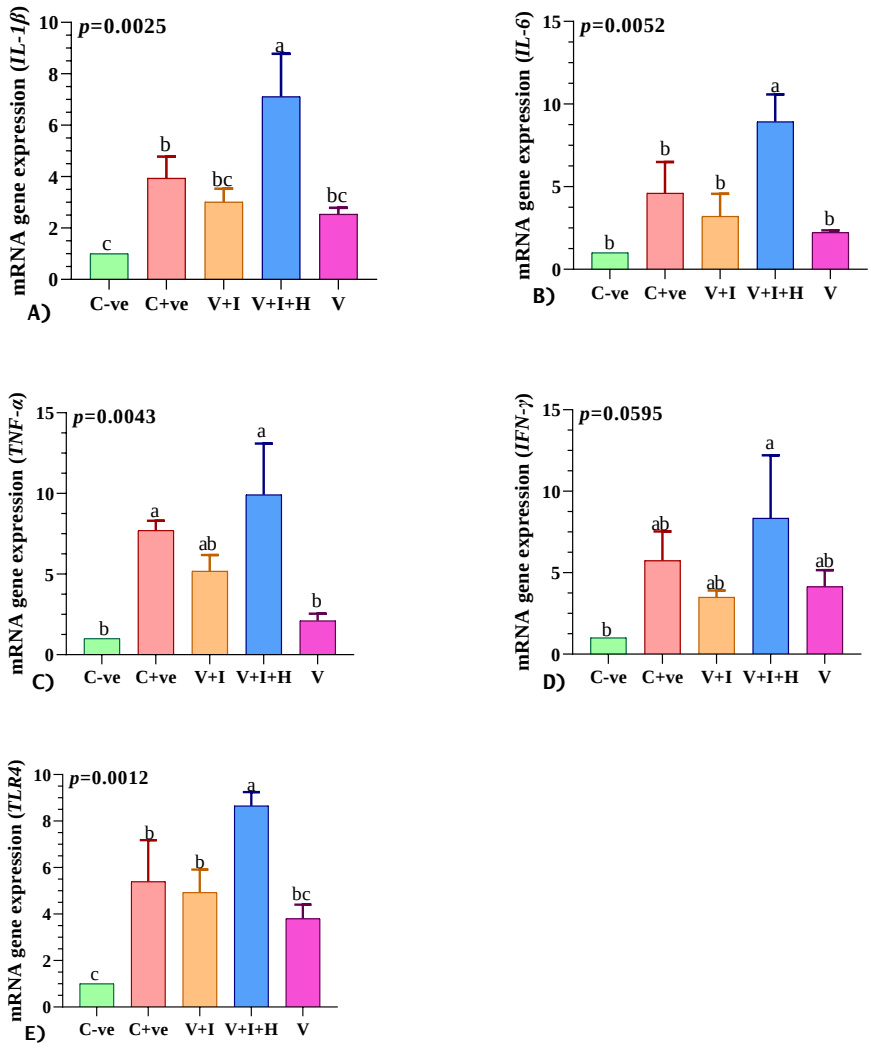

Fig. 2. Relative mRNA Expression Levels of Pro-inflammatory Cytokines (IL-1β, IL-6, TNF-α, and IFN-γ) and TLR-4 in Experimental Groups

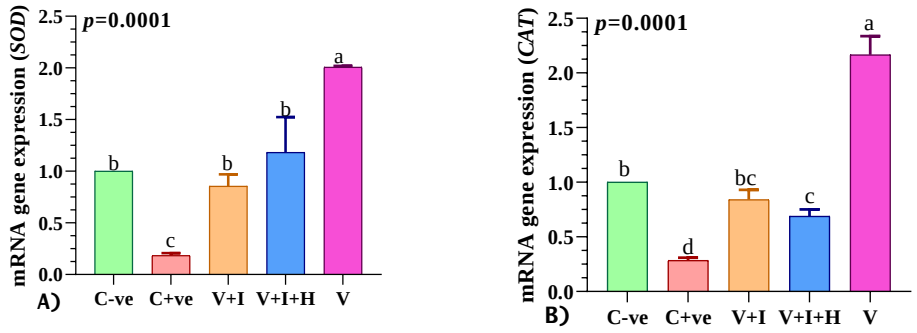

Fig. 3. Antioxidant Enzyme Gene Expression in Response to *E. coli* Challenge, Vaccination, and Heat Stress

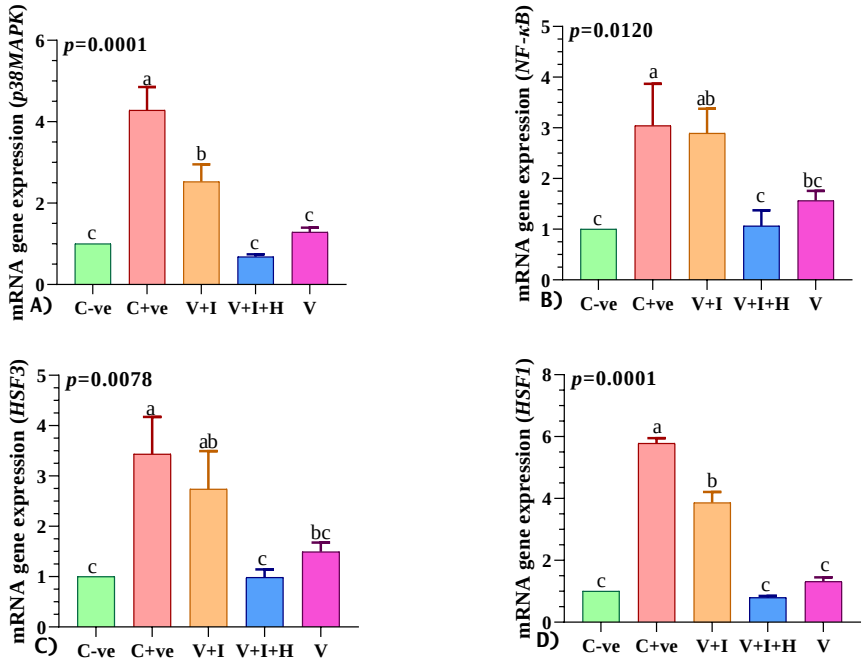

Fig. 4. Expression of Stress-Responsive Transcription Factors in Experimental Groups

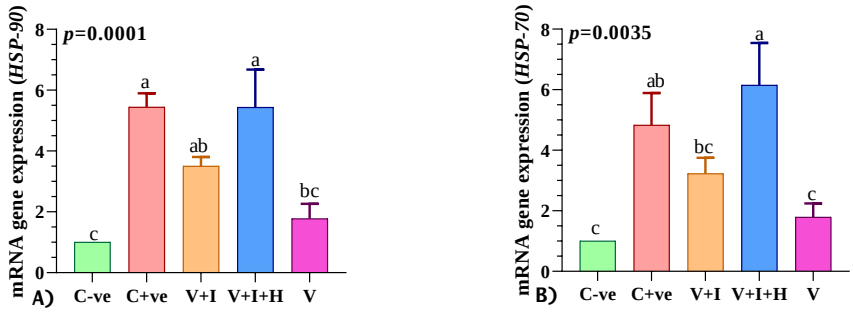

Fig. 5. Modulation of Heat Shock Protein (*HSP-90* and *HSP-70*) Gene Expression by Vaccination, *E. coli* Challenge, and Heat Stress

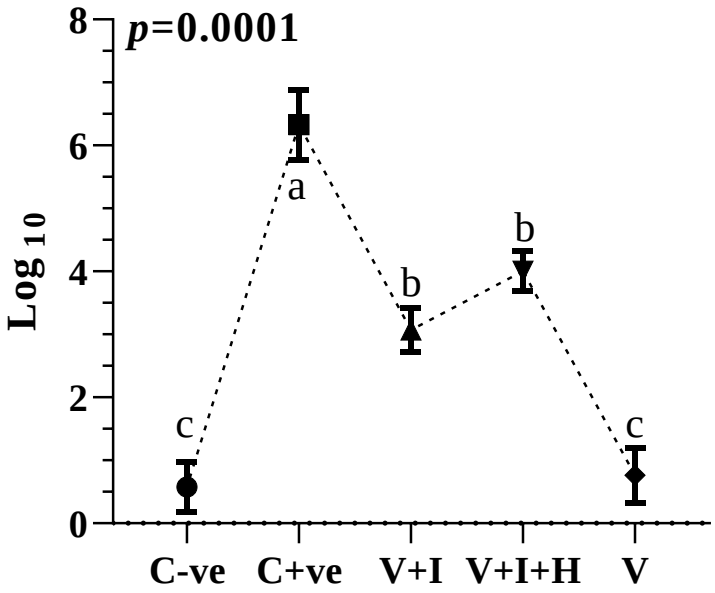

Fig. 6. Cecal *E. coli* Load in Experimental Groups

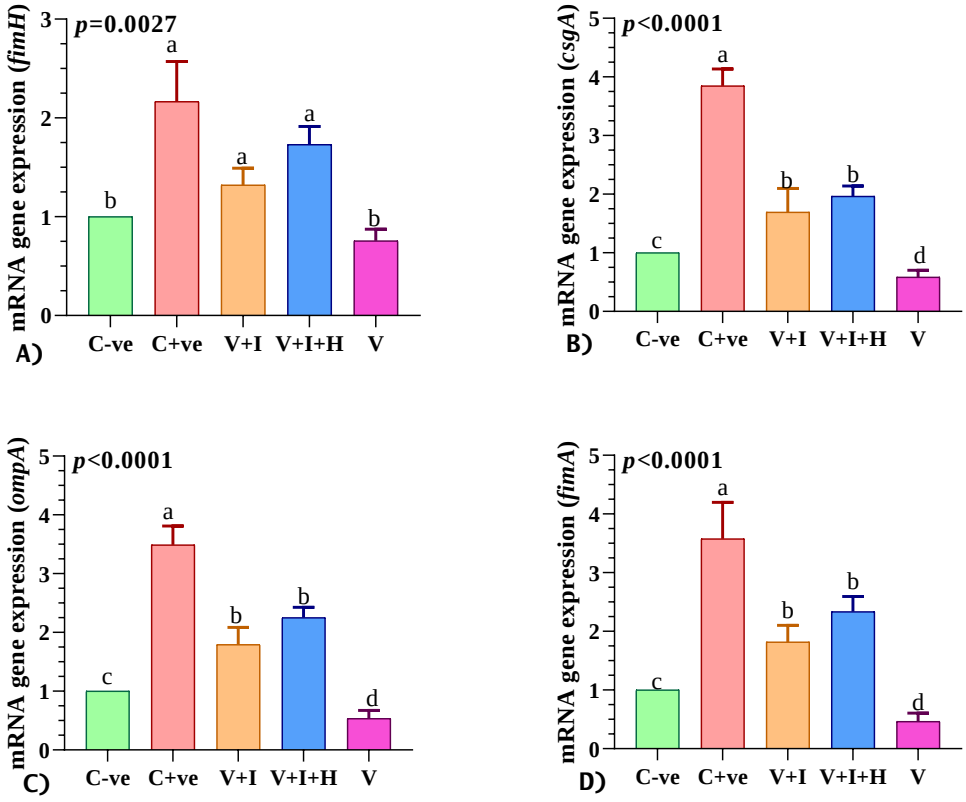

Fig. 7. The Expression of Biofilm-Related Genes in The Experimental Groups

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فك شيفرة التداخل الجزيئي بين الإجهاد الحراري، والتطعيم، وعدوى الإي كولاي في دجاج التسمين

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الملخص

تسبب عدوى الإشريكية القولونية (الإي كولاي) تهديدًا كبيرًا لصحة الدواجن، حيث يسهم تكوين الأغشية الحيوية بشكل كبير في تطور المرض. تتفرد هذه الدراسة بالتحقيق في التفاعلات المعقدة بين التطعيم، وعدوى الإشريكية القولونية، والإجهاد الحراري على دجاج اللاحم، مما يسد فجوة بحثية عادةً ما تدرس هذه العوامل بشكل منفرد. أثبت التطعيم فعالية عالية، حيث قلل بشكل كبير من معدل الوفيات والعلامات السريرية، وخفض بشكل ملحوظ حمل الإشريكية القولونية في الأعور لدى الطيور المصابة. على المستوى الجزيئي، أدى التطعيم إلى خفض تنظيم جميع الجينات الأربعة الرئيسية المرتبطة بالأغشية الحيوية، وعزز من التعبير عن إنزيمات مضادات الأكسدة.

كان أبرز ما توصلنا إليه هو وجود استجابة التهابية تآزرية وذات دلالة إحصائية في المجموعة التي تعرضت لثلاثة عوامل إجهاد (تم تطعيمها، وأُصيبت بالعدوى، وتعرضت للإجهاد الحراري)، حيث أظهرت أعلى تعبير للسيتوكينات المحفزة للالتهاب. تؤكد هذه النتائج على أهمية النظر في عوامل الإجهاد المتعددة في صحة الدواجن. وتوفر النتائج معلومات مباشرة لإدارة الدواجن من خلال دعم التطعيم كاستراتيجية شاملة لتعزيز صحه دجاج اللاحم، خاصة في المناطق المعرضة للإجهاد الحراري. علاوة على ذلك، تقدم هذه الدراسة خريطة طريق جزيئية لتطوير لقاحات من الجيل التالي لا تكتفي بتحفيز الاستجابة المناعية فحسب، بل تهيئ أيضًا الأنظمة الفسيولوجية للطائر للتكيف بشكل أفضل مع التحديات البيئية والمرضية مجتمعة.

الكلمات الدالة: التعبير الجيني للأغشية الحيوية، تعديل المناعة، صحة الدواجن، الاستجابة للإجهاد، التطعيم.