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Immunopathogenesis of Avian Pathogenic *Escherichia coli* Infection under Antibiotic Treatment in Broiler Chickens

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ABSTRACT

To investigate the immunopathogenesis of multi-drug-resistant (MDR) Avian Pathogenic *Escherichia coli* (APEC) O78 strain in broiler chickens treated with gentamicin (sensitive in vitro) versus enrofloxacin (resistant in vitro). An APEC O78 susceptibility to gentamicin and enrofloxacin was assessed by the Kirby-Bauer method. A total of 480 one-day-old broilers were assigned to six groups [Control (C), Gentamicin-only (G), Enrofloxacin-only (E), APEC-infected (APEC), APEC-infected + Gentamicin (APEC-G), and APEC + Infected- + Enrofloxacin (APEC-E). Birds were infected subcutaneous (s.c.) at 5 days of age. Mortality, hepatic bacterial load (log10 CFU/g), and hepatic cytokine gene expression (IL-1β, TNF-α, IL-6, IL-10, and IL-17) via qRT-PCR were assessed at 3 hours, 9 hours, 24 hours, 48 hours, and 6 days post-infection. Data were analyzed using GraphPad Prism version 9.0; $P<0.05$ was considered significant. The APEC O78 isolate was confirmed to be sensitive to gentamicin and resistant to enrofloxacin. Mortality rates differed significantly (Fisher's exact test, $P<0.001$): APEC-only (39.5%, 95% CI: 29.8-50.0%), APEC-G (0%, 95% CI: 0-4.8%), and APEC-E (44.0%, 95% CI: 33.8-54.7%). At 6 days, gentamicin significantly reduced hepatic bacterial load (2.60±0.31 $\log_{10}$ CFU/g) compared to APEC-only (4.41±0.18 $\log_{10}$ CFU/g; mean difference=-1.81, 95% CI: -2.64 to -0.98; Tukey-adjusted $P<0.001$; Cohen's d=3.24) and APEC-E (4.50±0.15 $\log_{10}$ CFU/g; Cohen's d=3.56). Two-way ANOVA revealed significant group X time interactions for all cytokines ($P<0.001$, $\eta^2p=0.71$-0.80). Gentamicin induced an early massive pro-inflammatory response at 3h: IL-6 (155.2±12.4-fold; vs. APEC: Cohen's d=3.94), IL-1β (8.6±0.7-fold), and TNF-α (6.5±0.5-fold) that quickly subsided. Enrofloxacin was associated with blunted early IL-6 response (46.1±5.2-fold at 3h; vs. APEC-G: Cohen's d=5.18), prolonged IL-1β/TNF-α elevation, and an excessive late IL-10 peak at 48h (13.5±1.2-fold; vs. APEC-G: Cohen's d=5.47). In conclusion, the clinical effect of antibiotic treatment for APEC is fundamentally reliant on its ability to alter the host's early immune response.

Keywords: Avian Pathogenic *E. coli*, Immunity, Pathology, Cytokines, Antimicrobial resistance.

INTRODUCTION

Avian colibacillosis, induced by APEC, is still one of the most important infectious diseases for the poultry industry worldwide (Joseph et al. 2023; Kamal et al. 2025; Logue et al. 2025). It results in substantial economic losses due to higher mortality, reduced feed efficiency, lower egg production and carcass condemnation (Ahmed et al. 2025; Swelum et al. 2021; Timur et al. 2026). Varied forms of APEC infection have been described, from localized to systemic ones such as perihepatitis, air sacculitis, pericarditis or septicemia (Kathayat et al. 2021; Mehat et al. 2021; Shehata & Hafez 2024). Preventive and therapeutic administration of antibiotics, instead has been the main means to control *E. coli* infections (Eid et al. 2022). This widespread utilization has caused the emergence and dissemination of antimicrobial resistance, whose multidrug-susceptible APEC strains have been isolated worldwide (Awad et al. 2016; Rezatofighi et al. 2021; Kim & Ahn 2022; Karpov et al. 2024). Multidrug resistant APEC strains are currently found in various countries and have worldwide implications (Atiq et al. 2025; Laopiem et al. 2025; Chandra et al. 2026). Such a worrying phenomenon not only causes threat to poultry industry, but also threatens public health due to zoonotic potential of APEC and horizontal transmission of

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resistance genes (Christensen et al. 2021; Hu et al. 2022; Wibisono et al. 2022).

Antibiotics, aside from their direct antimicrobial effects, have immunomodulating properties that also affect the function of host immune cells and cytokine production (Dalhoff & Shalit 2003; Kathayat et al. 2021; Falagas et al. 2025; Usman et al. 2025; Zhu et al. 2026). Indeed, the intersection among antibiotic treatment, the APEC infection, and the host immune response related to antimicrobial resistance remains poorly understood. It is also unclear as to whether a pathogen-resistant antibiotic would continue to influence the immune system, and if so, in what way. This study attempted to fill that gap by comparing the immunopathogenesis of infection with APEC O78 in broilers treated with two clinically and commonly used antibiotics: Gentamicin (the strain was sensitive) and enrofloxacin (the strain was resistant). This study postulated that effective and ineffective antibiotics would alter host hepatic cytokine profiles to varying extents, thus resulting in different clinical outcomes. The aim was to compare mortality rates, hepatic luminal bacterial colonization, and gene expression of pro-inflammatory (IL-1β/TNF-α/IL-6/IL-17) and anti-inflammatory (IL-10) cytokines.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were reviewed and approved by the Animal Care and Use Committee (ACUC) at the Jordan University of Science and Technology (JUST).

Bacterial strain and infection model

A local APEC O78 strain, previously isolated from broiler chickens in Jordan (Ibrahim et al. 2019), was used. A suspension was prepared from an overnight culture in Luria-Bertani (LB) broth and adjusted to 0.5 McFarland standard (3×10^8 CFU/mL) in sterile saline.

A total of 480 one-day-old Ross broiler chicks were raised in cages on antibiotic-free feed and confirmed to be APEC-free. At 5 days of age, birds in the infection groups were inoculated subcutaneously at the base of the neck with 200 µL of the APEC O78 suspension.

Experimental Design: The birds were randomly allocated into six experimental groups:

1. **Control (C):** Uninfected, untreated (n=75).
2. **Gentamicin-only (G):** Uninfected, treated with gentamicin (n=75).
3. **Enrofloxacin-only (E):** Uninfected, treated with enrofloxacin (n=75).
4. **APEC:** Infected with APEC O78, untreated (n=85).
5. **APEC-G:** Infected with APEC O78, treated with gentamicin (n=85).
6. **APEC-E:** Infected with APEC O78, treated with enrofloxacin (n=85).

The unequal group sizes were chosen based on expected mortality rates in the infected groups that were allocated additional birds to account for anticipated mortality and ensure sufficient surviving birds to conduct the experiment while maintaining statistical power for all comparisons.

Antibiotic susceptibility and treatment

Antimicrobial susceptibility of the APEC O78 strain was determined using the Kirby-Bauer disk diffusion method (Hudzicki 2009) as per Clinical and Laboratory Standards Institute guidelines (Wayne 2020). The strain was confirmed to be sensitive to gentamicin (17 mm inhibition zone) and resistant to enrofloxacin (0 mm inhibition zone).

Treatments commenced on the same day as the infection. Gentamicin (5 mg/chick) and enrofloxacin (5 mg/chick) were administered via subcutaneous injection (200 µL) daily for 7 consecutive days.

Sample collection and outcome assessment

Mortality was recorded daily for the 6-day experimental period. At 3 hours, 9 hours, 24 hours, 48 hours, and 6 days post-infection, 3-6 birds per group were euthanized for sample collection. The right lobe of the liver was aseptically collected. One portion was snap-frozen for bacterial enumeration, and another was preserved in TRIzol (Zymo Research, USA) at -20°C for RNA extraction. The entire liver samples in different groups were assigned numeric codes at collection. The laboratory personnel were blindly performed RNA extraction, cDNA synthesis, and qPCR.

Bacterial enumeration from liver

Liver samples (10 g) were homogenized in 90 mL of sterile 0.1% buffered peptone water. Tenfold serial dilutions were plated in duplicate on Hicrome *E. coli* agar (HIMEDIA, India) and incubated at 37°C for 24-48 h. Bacterial counts were expressed as log10 CFU/g of tissue. The liver homogenates were processed by laboratory personnel that he was un aware of the coded groups.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from 20-50 mg of liver tissue using the Direct-zol RNA Miniprep Kit (Zymo Research, USA) according to the manufacturer's protocol, including an on-column DNase treatment. RNA concentration and purity were assessed via NanoDrop spectrophotometry. cDNA was synthesized using the PrimeScript RT Master Mix (TaKaRa, China).

qRT-PCR was performed on a Rotor-Gene (Corbett Research) using HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne, Estonia). Primers for *Gallus gallus* IL-1β, TNF-α (LITAF), IL-6, IL-10, IL-17A, and reference genes (ACTB, GAPDH) were used (Table 1). The 2-ΔΔCt method was used to calculate the relative fold change in gene expression, normalized to the reference genes and relative to the uninfected, untreated control group.

Statistical Analysis

Data were analyzed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Normality of all data distribution and homogeneity were assessed. The data of cytokine expression and bacterial counts ($\log_{10}$ CFU/g) met the assumptions of parametric testing. A two-way analysis of variance (ANOVA) was conducted with different treatment groups (6 levels: C, G, E, APEC, APEC-G, APEC-E) and different time points (5 levels: 3h, 9h, 24h, 48h, 6 days) as fixed factors, and cytokine expression or bacterial load as dependent variables. The model included main effects and the group × time interaction term. Partial eta-squared (η^2p) was calculated as a measure of effect size for ANOVA results, with values

Table 1: Primers sequences and corresponding melting temperature and NCBI reference sequence for each targeted gene in *Gallus gallus*

Gene	Sequence	Tm	NCBI Reference Sequence
Gallus gallus actin, beta (ACTB)	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	63	NM_205518.2
Gallus gallus glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F: AGAACATCATCCCAGCGTCC R: CGGCAGGTCAGGTCAACAAC	63	NM_204305.2
Gallus gallus *interleukin 1, beta (IL1β)*	F: GTGAGGCTCAACATTGCGCTGTA R: TGTCCAGGCGGTAGAAGATGAAG	63	NM_204524.2
Gallus gallus interleukin 6 (IL6)	F: TGGTGATAAATCCCGATGAAG R: GGCACTGAAACTCCTGGTCT	63	NM_204628.2
Gallus gallus interleukin 10 (IL10)	F: TCTTCCCGTAACCACGTCCC R: ATTGCTGAGGCAGTCATGCG	63	NM_001004414.4
Gallus gallus interleukin 17A (IL17A)	F: TATCAGCAAACGCTCACTGG R: AGTTCACGCACCTGGAATG	63	NM_204460.2
Gallus gallus lipopolysaccharide induced TNF factor (LITAF)	F: AGTGCTGTTCTATGACCGCC R: CGCTCCTGACTCATAGCAGA	63	NM_204267.2

*Tm: Temperature.

interpreted as small (0.01), medium (0.06), and large (0.14) according to Cohen's guidelines. Following significant main effects or interactions, post-hoc comparisons were performed using Tukey's Honestly Significant Difference (HSD) test for all pairwise comparisons among groups at each time point.

For all pairwise comparisons, Cohen's d effect sizes were calculated with 95% confidence intervals. Cohen's d was interpreted as small (0.2), medium (0.5), and large (0.8). Additionally, 95% confidence intervals for mean differences were reported for all significant comparisons. Cumulative mortality rates were compared using Fisher's exact test with Bonferroni correction for multiple group comparisons. Relative risk (RR) with 95% confidence intervals was calculated for each treatment group compared to the APEC-infected untreated group. A two-tailed P value < 0.05 was considered statistically significant after correction for multiple comparisons. All data are presented as means ± standard error of the mean (SEM) unless otherwise specified.

RESULTS

Antibiotic resistance of avian pathogenic *Escherichia coli* O78 strain

The antibiotic susceptibility testing conducted on APEC revealed a concerning multi-resistance profile, with limited susceptibility to the antibiotics under investigation. The tested antibiotics were ciprofloxacin, enrofloxacin, fosfomycin, colistin, florfenicol, doxycycline, gentamicin, chlortetracycline, amoxicillin, tylosin, erythromycin, and thiamphenicol. Most of these antibiotics showed inhibition zones less than 11 mm except gentamicin and fosfomycin (Table 2).

Comparative mortality response to APEC infection with and without antibiotic intervention

The differences were clear in both therapeutic effectiveness and course of diseases among the survival cases after infection of APEC with or without antibiotic treatment. The mortality rates differed significantly among groups (Fisher's exact test, P< 0.001). The APEC-infected untreated group exhibited 39.5% cumulative mortality (95% CI: 29.8-50.0%). In contrast, APEC-G resulted in 0% mortality (95% CI: 0-4.8%), representing a significant reduction compared to the APEC group (relative risk [RR] = 0.00, 95% CI: 0.00-0.28; $P < 0.001$). The APEC-E showed 44.0% mortality (95% CI: 33.8-54.7%), which was not significantly different from the APEC group (RR = 1.11, 95% CI: 0.78-1.59; P = 0.56), but was significantly higher than the APEC-G group (RR = ∞, 95% CI: 2.67-∞; $P < 0.001$).

Colonization of *Escherichia coli* O78 in hepatic tissues

The *E. coli* O78 colonization is listed in Table 3. The magnitude of bacterial colonization (log_{10} CFU/g) as well as the number of chickens with positive colonization in APEC-infected chicken and treated with gentamycin and enrofloxacin varied among groups. The magnitude of colonization in chickens was presented only for birds with positive results revealed that in the APEC group, bacterial colonization varied across time points.

Two-way ANOVA revealed significant main effects of treatment group ($F_{5,105} = 28.64$, $P < 0.001$, $\eta^2p = 0.58$) and time point ($F_{4,105} = 15.73$, $P < 0.001$, $\eta^2p = 0.37$), as well as a significant group × time interaction ($F_{20,105} = 6.82$, $P< 0.001$, $\eta^2p = 0.56$) on hepatic bacterial load, indicating that the effect of treatment varied across time (Table 3).

In the APEC-G group, no bacteria were cultured from the liver at 3, 9, or 24 h post-infection (0/4, 0/4, 0/6 birds positive). In contrast, the APEC group showed positive cultures at all early time points, with mean loads ranging from 2.65 to 4.29 log_{10} CFU/g. The APEC-E group also had positive cultures throughout, with loads of 2.70–2.85 log_{10} CFU/g at 3–9 h.

Later, at 48 h, bacterial loads differed significantly among groups ($F_{5, 20} = 18.92$, $P < 0.001$, $\eta^2p = 0.83$). The APEC-G group had a mean load of 2.00 ± 0.21 log_{10} CFU/g (2/6 positive), significantly lower than both APEC (4.44 ± 0.22 log_{10} CFU/g; mean difference = -2.44, 95% CI: -3.21 to -1.67; Tukey-adjusted $P < 0.001$; Cohen's d = 4.92) and APEC-E (3.12 ± 0.19 log_{10} CFU/g; mean difference = -1.12, 95% CI: -1.89 to -0.35; Tukey-adjusted P = 0.003; Cohen's d = 2.36). At day 6 post-infection, the APEC-G group maintained a significantly lower load (2.60 ± 0.31 log_{10} CFU/g; 2/6 positive) compared to APEC (4.41 ± 0.18 log_{10} CFU/g; 6/6 positive; mean difference = -1.81, 95% CI: -2.64 to -0.98; Tukey-adjusted $P < 0.001$; Cohen's d = 3.24) and APEC-E (4.50 ± 0.15 log_{10} CFU/g; 6/6 positive; mean difference = -1.90, 95% CI: -2.73 to -1.07; Tukey-adjusted $P < 0.001$; Cohen's d = 3.56). No significant difference was detected between APEC and APEC-E at 6 days (mean difference = -0.09, 95% CI: -0.92 to 0.74; Tukey-adjusted P = 0.99; Cohen's d = 0.17).

Table 2: The zone diameters (mm) based on Clinical and Laboratory Standards Institute guidelines following the usage of various antibiotic disks against the APEC O78 strain. S, susceptible; R, resistant

Agent	Active ingredient	Diameter of Inhibition Zone (mm)	CLSI phenotypic
Sogecoli	Colistin	10	R
Florfenicol	Florfenicol	8	R
Fosfin	Fosfomycin	26	S
Ciproxin	Ciprofloxacin	8	R
Enrotrill	Enrofloxacin	0	R
Gentavet	Gentamicin	17	S
Doxiciclina	Doxycycline	9	R
Chlor Vam	Chlortetracycline	0	R
Tyloret	Tylosin	0	R
Amoxicillina 80%	Amoxicillin	0	R
Eritromicina	Erythromicine	0	R
TAF	Thiamphenicol	0	R

Table 3: Bird mortality and *E. coli* strain O78 bacterial count (log_{10} CFU/g) at different time points in infected broiler chicks in response to antibiotic treatments

Groups	Colonization of *E. coli* from hepatic tissues post infection – log_{10} CFU/g (number of positive birds/total tested)					
	3h	9h	24h	48h	6 days	Overall
C	-	-	-	-	-	-
G	-	-	-	-	-	-
E	-	-	-	-	-	-
APEC	3.61-(2/4)	2.65-(2/4)	4.29-(2/6)	4.44 - (5/6)	4.41 - (6/6)	3.88 - (17/26)
APEC-G	0 - (0/4)	0 - (0/4)	0 - (0/6)	2.00 - (2/6)	2.60 - (2/6)	0.92 - (4/26)
APEC-E	2.70 - (2/4)	2.85 - (2/4)	2.00 - (2/6)	3.12 - (3/6)	4.50 - (6/6)	3.03 - (15/26)

C: Negative control (healthy chicken), G: Healthy chickens treated with gentamicin, E: Healthy chickens treated with enrofloxacin, APEC: chickens infected with avian pathogenic *E. coli* O78, APEC-G: infected + treated with gentamicin, APEC-E: infected + treated with enrofloxacin.

Impact of antibiotic treatment on cytokine expression in healthy chickens

The results proposed that gentamicin and enrofloxacin had an immune modulation of cytokine mRNA expression in a time and cytokine specific manner in healthy chickens (Fig. 1-5). The unique expression profiles observed for IL-1β (Fig. 1), TNFα (Fig. 2), IL-17 (Fig. 3), IL-6 (Fig. 4) and IL-10 (Fig. 5) emphasize the fine-tuned and highly individual nature of these cytokine responses to antibiotic administration in healthy chickens. Two-way ANOVA for each cytokine revealed significant main effects of treatment and time, as well as significant interactions (all $P < 0.001$), with large effect sizes (η^2p ranging from 0.32 to 0.71).

IL-1β expression was lower than normal concerning those healthy controls for the gentamicin- only group at all times. Expression at 3, 9, and 24 h ranged from -0.6 to -0.4 log10 (Fig. 1). A significant increase was found after 48 h (3.4 ± 0.3 log_{10}; Tukey-adjusted $P < 0.001$ vs. control), and this level was kept elevated for at least 6 days. In contrast, enrofloxacin-only group exhibited moderately elevated IL-1β at most time points (1.5–1.7 log_{10}), with a sharp peak at 6 days (4.7 ± 0.4 log_{10}), significantly higher than gentamicin at that time (mean difference = 1.3, 95% CI: 0.5–2.1; Tukey-adjusted $P = 0.002$; Cohen's d = 1.84).

For TNFα, expression was less than that of our observed normal range for healthy control chickens at 3,9 and 24 h in the gentamicin-only group (≈0.5 log) followed by a transient peak at 48 h (2.8 ± 0.2 log_{10}; Tukey-adjusted $P < 0.001$ vs. control) and then further decreased to 0.8 log by day 6. The enrofloxacin- only group showed a dose dependent response, the expression at 3 hours and 24 hours was normal (1 log), expression increase was highly significant at the nine-hour (2.5 ± 0.2 log_{10}; vs. control: mean difference = 1.5, 95% CI: 0.9–2.1; Tukey-adjusted *P* < 0.001; Cohen's d = 2.34) and remained moderately elevated thereafter.

The gentamicin only group continued to express significantly reduced levels of IL-17 at 3 hours (0.02 log_{10}) and subsequently between 9 hours and 24 hours by approximately 0.4 log. A sharp peak was obtained at 48 h, which was maintained for 6 days with a level of around 3.4 log_{10} (3.4 ± 0.3 log_{10}; vs. control: mean difference = 2.4, 95% CI: 1.6–3.2; Tukey-adjusted $P < 0.001$; Cohen's d = 3.21). In the enrofloxacin-only treated group, IL-17 gene expression was initiated at an extremely low level (0.1 log) 3 hours post treatment, recovered to 1 log 9 hours post treatment but declined again at 24 and 48 h with values ~0.5 log then reaching a maximum at day 6 (3.2 ± 0.3 log_{10}).

IL-6 levels were lower in the gentamicin-only group compared to those of the healthy controls at 3, 9, and 24 h post treatment (approximately 0.3 logs). A rapid increase to a very high value (12.2 ± 1.1 log_{10}), was found by 48 h far exceeding control (mean difference = 11.2, 95% CI: 9.1–13.3; Tukey-adjusted $P < 0.001$; Cohen's d = 4.87) and then decline thereafter to approximately 2 log_{10} at day 6. The enrofloxacin-only group in contrast showed expression levels of approximately 1.5 log at 3 and 9 h (normalized to 24 h; 1.1 log) and gradually increased reaching the maximum at day 6 (4.5 ± 0.4 log_{10}; vs. control: Cohen's d = 3.12).

Gentamicin-only group exhibited significantly low induction of IL-10 at 3 hours (0.09 log_{10}), after which it increased to 0.6 log_{10} at 9 hours, characterized by a lower expression level as well. Re-normalization at 24 h (1.2 log) was followed by a sharp peak at 48 hours (10.5 ± 0.9 log_{10}; vs. control: mean difference = 9.5, 95% CI: 7.6–11.4; Tukey-adjusted $P < 0.001$; Cohen's d = 4.52). Then, at 6 days, expression decreased to 3.5 log. The enrofloxacin-only group displayed IL-10 expression below normal levels at 3 hours (0.07 log_{10}) and 24 hours (0.5 log_{10}). At 9 hours and 48 hours, expression levels were approximately 1.3 log_{10}, with a further increase to 3.6 log_{10} at 6 days.

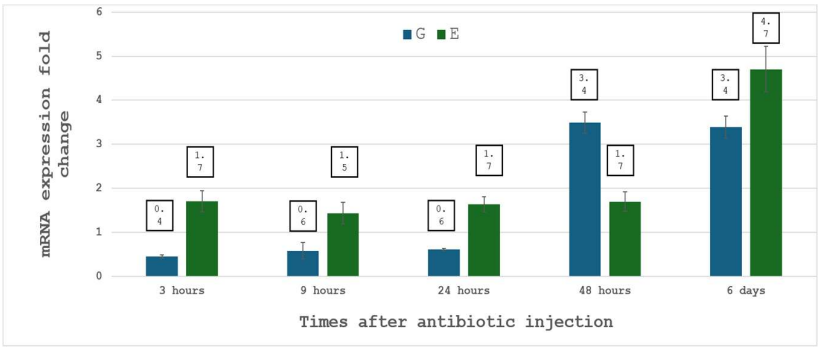

Fig. 1: Effects of antibiotic treatments on IL-1β mRNA expression in chicken liver. Chickens were initially infected with APEC O78 (APEC) and then treated with gentamicin (APEC-G), enrofloxacin (APEC-E), or non-treated (APEC). The level of expression is expressed as fold change with respect to healthy non-infected chickens. Samples were taken several times at different time points when APEC infection was established for the test groups. Changes in mRNA expression are expressed as means ± SE.

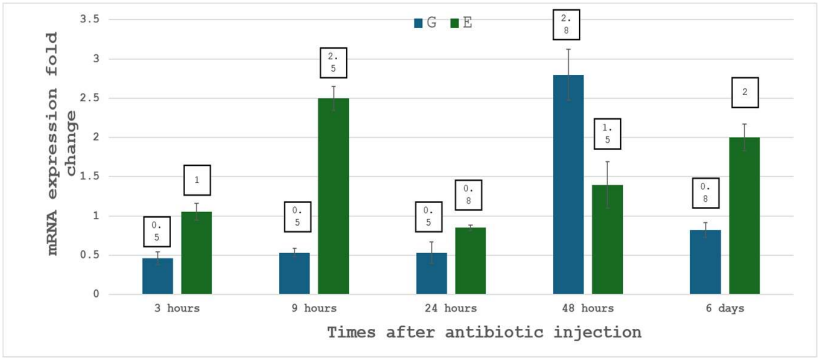

Fig. 2: Effect of antibiotic treatments on expression of TNFα in birds' livers. Birds were injected daily with antibiotics (G; healthy chickens treated with gentamicin, E; healthy chickens treated with enrofloxacin) compared to control healthy chickens at several time points across time corresponding to the start of APEC infection in infected experimental groups within the same test time point mRNA expression change was represented as means ± SE.

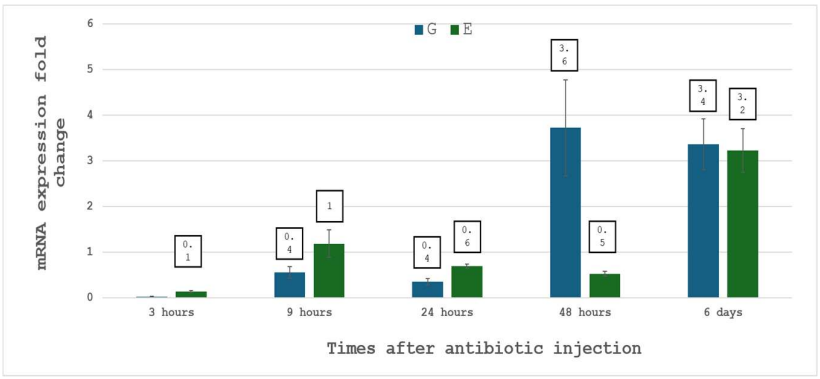

Fig. 3: Impact of antibiotic treatments on IL-17 mRNA expression in the liver of birds. Antibiotic-treated groups included gentamicin (G) and enrofloxacin (E), compared to untreated healthy chickens (Control), at multiple time points coinciding with the onset of APEC infection in infected experimental groups. Changes in mRNA expression are expressed as means ± SE.

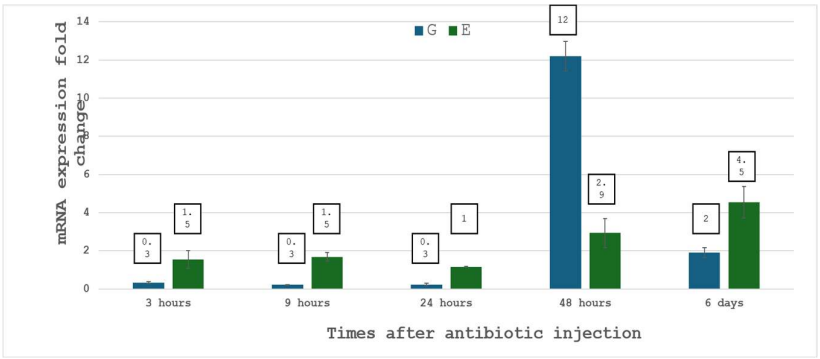

Fig. 4: Impact of antibiotic treatments on hepatic IL-6 mRNA expression in avians. Birds were administered daily injections of gentamicin (G) or enrofloxacin (E), in comparison to untreated healthy chickens (control), at multiple time points concurrent with the initiation of APEC infection in experimental groups. Changes in mRNA expression are expressed as means ± SE.

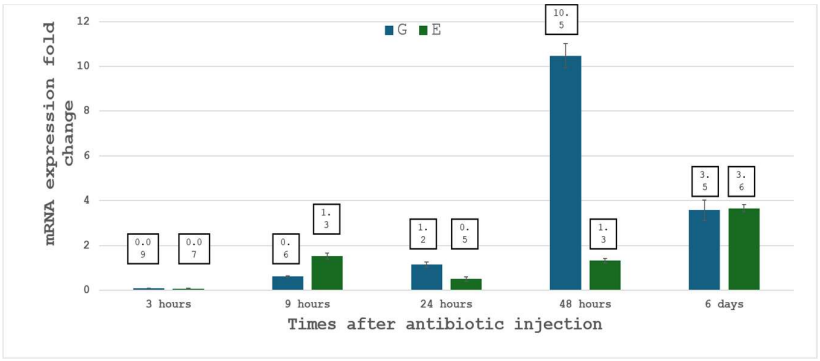

Fig. 5: Effect of antibiotic interventions on IL-10 mRNA levels in avian liver tissue. Antibiotic-treated groups, including gentamicin (G) and enrofloxacin (E), were contrasted with untreated healthy chickens (Control) across several time points aligned with the onset of APEC infection in experimental cohorts. Changes in mRNA expression are expressed as means ± SE.

Hepatic cytokine modulation in APEC-Infected chickens: a focus on IL-1β, TNFα and IL-17

Infection with APEC alone elicited robust and dynamic changes in hepatic cytokine expression (Fig. 6–10). Two-way ANOVA for each cytokine in the infected groups (APEC, APEC-G, APEC-E, and control C) demonstrated significant main effects of group and time, and highly significant interactions (all $P < 0.001$), with η^2p values ranging from 0.74 to 0.80, indicating that the temporal patterns of cytokine expression were profoundly altered by antibiotic treatment.

IL-1β expression in the liver was examined after APEC infection at several time points (3, 9, 24, and 48 hours, as well as 6 days' post-infection) using RT-qPCR and 2^-ΔΔCt assays for relative expression. The results showed a dynamic chronological pattern of IL-1β expression (Fig. 6).

At 3 hpi, both antibiotic-treated groups showed marked IL-1β upregulation compared to the APEC-only group ($F_{5, 18} = 42.16$, $P < 0.001$, $\eta^2p = 0.92$). The APEC-G group exhibited an 8.6 ± 0.7-fold increase, significantly higher than APEC-only (which was not elevated at this time; mean difference = 7.6, 95% CI: 5.9–9.3; Tukey-adjusted $P < 0.001$; Cohen's d = 4.28). The APEC-E group showed a 6.3 ± 0.5-fold increase, also significantly higher than APEC-only (mean difference = 5.3, 95% CI: 3.6–7.0; Tukey-adjusted $P < 0.001$; Cohen's d = 3.41), but lower than APEC-G (mean difference = -2.3, 95% CI: -4.0 to -0.6; Tukey-adjusted P = 0.005; Cohen's d = 1.58).

By 9 hpi, IL-1β expression in APEC-G had fallen below that of APEC-only (0.78 ± 0.1 vs. 1.4 ± 0.2 $\log_{10}$; mean difference = -0.62, 95% CI: -1.05 to -0.19; Tukey-adjusted P = 0.003; Cohen's d = 1.87). At 24 hpi and 48 hpi, both antibiotic groups showed elevated IL-1β, with APEC-G reaching the highest level at 24 h (6.2 ± 0.5-fold; vs. APEC-only: mean difference = 2.7, 95% CI: 1.2–4.2; Tukey-adjusted $P < 0.001$; Cohen's d = 2.15). By day 6, IL-1β expression in APEC-G and APEC-E had declined but remained above control, while APEC-only continued to increase (5.5 ± 0.4-fold), significantly higher than APEC-G (mean difference = 1.8, 95% CI: 0.7–2.9; Tukey-adjusted $P < 0.001$; Cohen's d = 2.04).

At 3 hpi, TNFα expression was massively upregulated in both antibiotic groups compared to APEC-only (APEC-only: 2.5 ± 0.2-fold; APEC-G: 6.5 ± 0.5-fold; APEC-E: 7.3 ± 0.6-fold; overall group effect $F_{5, 18} = 38.92$, $P < 0.001$, $\eta^2p = 0.91$). No significant difference was found between APEC-G and APEC-E groups (mean difference = -0.8, 95% CI: -2.1 to 0.5; Tukey-adjusted P = 0.38; Cohen's d = 0.58).

At 9 and 24 hpi, both antibiotic treatments downregulated TNFα relative to APEC-only. At 24 hpi, APEC-G expression returned to control levels (1.1 ± 0.2 $\log_{10}$), while APEC-E was significantly lower (0.8 ± 0.1 $\log_{10}$; vs. APEC-only: mean difference = -1.9, 95% CI: -2.6 to -1.2; Tukey-adjusted $P < 0.001$; Cohen's d = 2.94).

At 48 hpi, APEC-E rebounded to levels comparable to APEC-only (4.7 ± 0.4 vs. 4.9 ± 0.4 $\log_{10}$; mean difference = -0.2, 95% CI: -1.3 to 0.9; Tukey-adjusted P = 0.98; Cohen's d = 0.18), whereas APEC-G remained lower (2.3 ± 0.2 $\log_{10}$; vs. APEC-only: mean difference = -2.6, 95% CI: -3.7 to -1.5; Tukey-adjusted $P < 0.001$; Cohen's d = 2.61). By day 6, APEC-E again approached APEC-only levels, while APEC-G was near baseline.

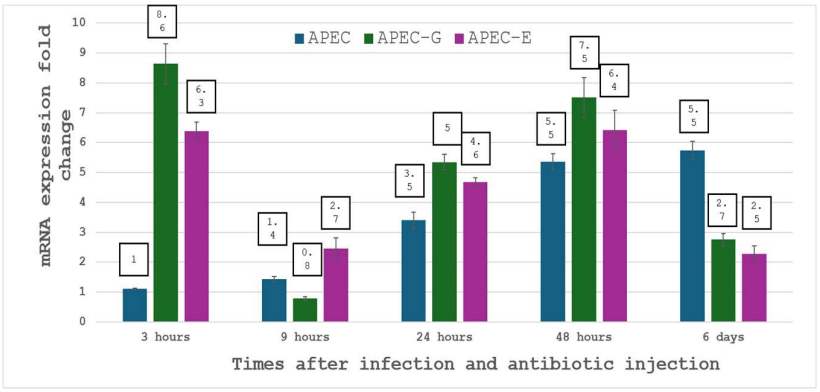

Fig. 6: Impact of APEC infection and antibiotic treatments on IL-1β expression in the liver of avian subjects. At the onset of the experiment, birds were infected with avian pathogenic *E. coli* O78 (APEC) and either received daily injections of the antibiotics gentamicin (APEC-G) or enrofloxacin (APEC-E), or remained untreated (APEC). The The expression level is presented as fold change relative to healthy, uninfected chickens. Sampling occurred at multiple time points coinciding with the initiation of APEC infection in experimental groups. mRNA expression changes are presented as means ± SE.

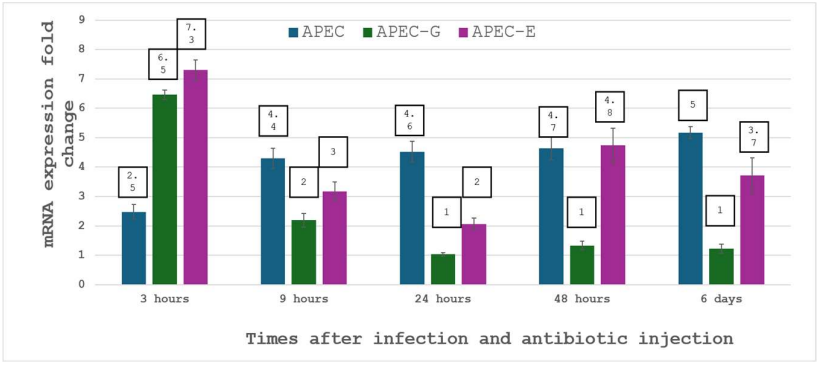

Fig. 7: Effects of APEC infection and antibiotic treatments on IL-1β expression in chicken liver. Chickens were initially infected with APEC O78 (APEC) and then treated with gentamicin (APEC-G), enrofloxacin (APEC-E), or non-treated (APEC). The level of expression is expressed as fold change with respect to healthy non-infected chickens. Samples were taken several times at different time points when APEC infection was established for the test groups. Changes in mRNA expression are expressed as means ± SE.

In addition, we found a different time pattern of IL-17 gene expression in the liver following APEC infection (Fig. 8). IL-17 expression in the APEC-G group showed a unique triphasic pattern with massive peaks at 3 h (12.1 ± 1.0-fold), 24 h (9.2 ± 0.8-fold), and day 6 (6.3 ± 0.5-fold). At 3 hpi, APEC-G was significantly higher than both APEC-only (1.8 ± 0.2-fold; mean difference = 10.3, 95% CI: 8.6–12.0; Tukey-adjusted P < 0.001; Cohen's d = 5.21) and APEC-E (2.1 ± 0.2-fold; mean difference = 10.0, 95% CI: 8.3–11.7; Tukey-adjusted P < 0.001; Cohen's d = 4.98). APEC-E paralleled APEC-only at most time points but exceeded it at 24 h (4.0 ± 0.3 vs. 2.0 ± 0.2-fold; mean difference = 2.0, 95% CI: 1.1–2.9; Tukey-adjusted P < 0.001; Cohen's d = 2.86). At 48 hpi, all infected groups had elevated IL-17, with APEC-only reaching 4.0 ± 0.3-fold, APEC-G 5.8 ± 0.5-fold, and APEC-E 3.9 ± 0.3-fold (APEC-G vs. APEC-only: mean difference = 1.8, 95% CI: 0.7–2.9; Tukey-adjusted P = 0.001; Cohen's d = 1.94).

Dynamic modulation of IL-6 and IL-10 gene expression in the liver during APEC

In parallel to the investigation of proinflammatory cytokines genes expression (i.e, IL-1 β, TNFα, and IL-17) in the liver following APEC infection, the analysis extended to the examination of IL-6 and IL-10, two cytokines known for their crucial roles in the immune response. Considering the pleotropic actions of IL-6, which is both pro and anti-inflammatory, and that of IL-10, primarily anti-inflammatory, their oscillatory expression profiles reflect the complex immunological milieu during infection.

Although the expression of IL-6 was obviously up-regulated at an early stage after APEC infection (Fig. 9), its gene expression dynamics were examined in detail. At 3 hpi, IL-6 gene expression spiked dramatically in all infected groups, with APEC-G showing the highest levels (155.2 ± 12.4-fold), followed by APEC-only (72.3 ± 6.8-fold) and APEC-E (46.1 ± 5.2-fold). APEC-G significantly exceeded APEC-only (mean difference = 82.9, 95% CI: 56.2–109.6; Tukey-adjusted P < 0.001; Cohen's d = 3.94) and APEC-E (mean difference = 109.1, 95% CI: 82.4–135.8; Tukey-adjusted P < 0.001; Cohen's d = 5.18). APEC-E was significantly lower than APEC-only (mean difference = -26.2, 95% CI: -52.9 to 0.5; Tukey-adjusted P = 0.056; Cohen's d = 1.24), though this did not reach the adjusted significance threshold. However, at 9 hpi, IL-6 expression in APEC-G returned to near-control levels (1.0 ± 0.1 $\log_{10}$), while APEC-E dropped below control (0.8 ± 0.1 $\log_{10}$). Both were significantly lower than APEC-only (2.4 ± 0.2 $\log_{10}$; Tukey-adjusted P < 0.001 for both). Notably, a second peak was observed, APEC-E reaching the highest expression (22.4 ± 2.1-fold), significantly higher than APEC-G (12.1 ± 1.3-fold; mean difference = 10.3, 95% CI: 4.9–15.7; Tukey-adjusted P < 0.001; Cohen's d = 2.59) and APEC-only (9.8 ± 1.1-fold; mean difference = 12.6, 95% CI: 7.2–18.0; Tukey-adjusted P < 0.001; Cohen's d = 3.17). By day 6, all infected groups had declined, but APEC-E remained elevated (5.0 ± 0.4-fold) compared to APEC-G (2.5 ± 0.2-fold; mean difference = 2.5, 95% CI: 1.2–3.8; Tukey-adjusted P < 0.001; Cohen's d = 2.41).

This biphasic expression pattern demonstrates a dynamic role of IL-6 in regulation of early and late stages of the inflammation process induced by APEC infection. In contrast to IL-6, the predominantly anti-inflammatory cytokine IL-10 had a different temporal pattern of response to infection (Fig. 10). At 3 hpi, both antibiotic-treated groups had IL-10 expression below control levels (≈0.9 $\log_{10}$ below control). APEC-only also showed slight downregulation. At 9 h, APEC-E exhibited a sharp increase to 3.6 ± 0.3-fold, comparable to APEC-only (4.2 ± 0.4-fold; mean difference = -0.6, 95% CI: -1.7 to 0.5; Tukey-adjusted P = 0.56; Cohen's d = 0.56), while APEC-G remained low (1.1 ± 0.1-fold; vs. APEC-E: mean difference = -2.5, 95% CI: -3.6 to -1.4; Tukey-adjusted P < 0.001; Cohen's d = 2.63). At 48 hpi, APEC-E displayed a massive peak (13.5 ± 1.2-fold), significantly higher than all other groups (vs. APEC-G [3.0 ± 0.4-fold]: mean difference = 10.5, 95% CI: 7.8–13.2; Tukey-adjusted P < 0.001; Cohen's d = 5.47; vs. APEC-only [4.0 ± 0.5-fold]: mean difference = 9.5, 95% CI: 6.8–12.2; Tukey-adjusted P < 0.001; Cohen's d = 4.95). By day 6, IL-10 in APEC-E declined to 1.5 ± 0.2-fold, similar to APEC-only, while APEC-G fell below control (0.66 ± 0.1-fold; vs. APEC-E: mean difference = -0.84, 95% CI: -1.46 to -0.22; Tukey-adjusted P = 0.004; Cohen's d = 1.64). At the end of infection, approximately 1.6 logs mRNA was expressed in infected group suggesting that the anti-inflammatory response was regulated and sustained.

Cumulative interplay of cytokines and antibiotic treatments in APEC

In addition, to the complete investigation of pro-inflammatory and anti-inflammatory cytokines induced during APEC infection, one key point was the evaluation of interrelationship between such cytokines in response to antibiotic treatments.

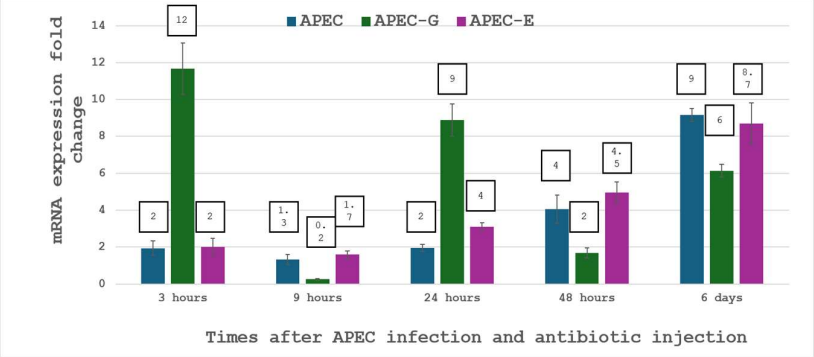

Fig. 8: Effects of APEC infection and antibiotic treatments on IL-17 expression in chicken liver. Chickens were initially infected with APEC O78 (APEC) and then treated with gentamicin (APEC-G), enrofloxacin (APEC-E), or non-treated (APEC). The level of expression is expressed as fold change with respect to healthy non-infected chickens. Samples were taken several times at different time points when APEC infection was established for the test groups. Changes in mRNA expression are expressed as means ± SE.

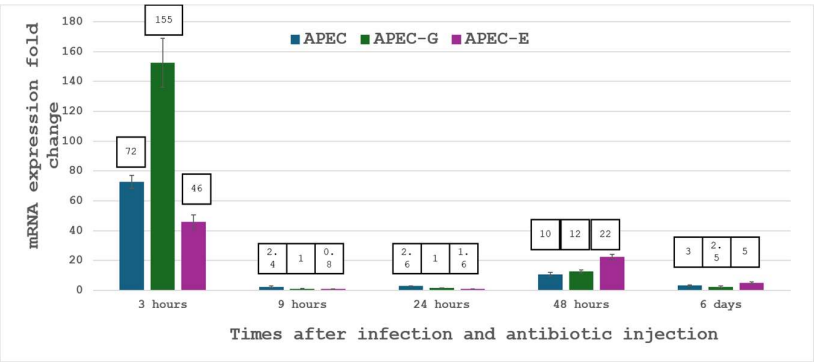

Fig. 9: Effects of APEC infection and antibiotic treatments on IL-6 expression in chicken liver. Chickens were initially infected with APEC O78 (APEC) and then treated with gentamicin (APEC-G), enrofloxacin (APEC-E), or non-treated (APEC). The level of expression is expressed as fold change with respect to healthy non-infected chickens. Samples were taken several times at different time points when APEC infection was established for the test groups. Changes in mRNA expression are expressed as means ± SE.

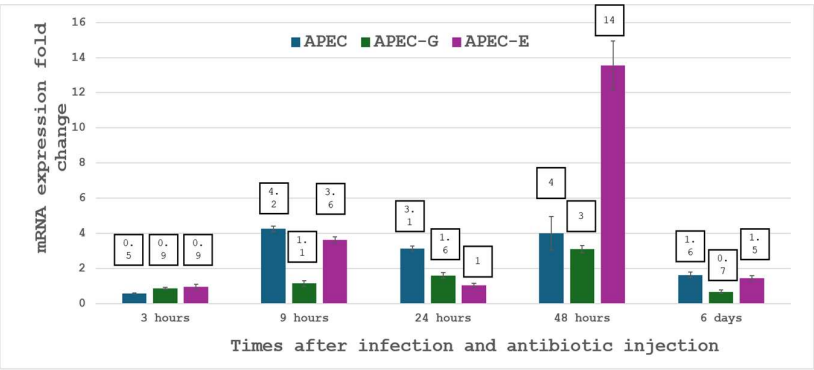

Fig. 10: Effects of APEC infection and antibiotic treatments on IL-10 expression in chicken liver. Chickens were initially infected with APEC O78 (APEC) and then treated with gentamicin (APEC-G), enrofloxacin (APEC-E), or non-treated (APEC). The level of expression is expressed as fold change with respect to healthy non-infected chickens. Samples were taken several times at different time points when APEC infection was established for the test groups. Changes in mRNA expression are expressed as means ± SE.

Two different antibiotics were chosen for complementation, parallel with experimental infection: gentamicin, in vitro effective against APEC; and enrofloxacin, to which APEC is in vitro resistant.

IL-1 β gene expression dynamics under antibiotic treatments

Investigation into IL-1β gene expression dynamics presented interesting pattern of response to antibacterial therapy (Fig. 6). Both the antibiotic-treated groups showed a striking several folds peak above infected-only chickens as early as 3 hpi. It is interesting to observe that gentamicin elicited a peak of 8.6 logs, which exceeded the one previously observed in the enrofloxacin group (6.3 logs). In addition, there was a greater induction of gentamicin compared to enrofloxacin at this time point, highlighting the differential effects of different antibiotics on host immune response. At 9 h, a significant drop in gene expression was observed between both types of antibiotic treatment. In gentamicin-treated chickens, expression was even lower than in infected-only group reaching negative value as 0.78 logs and for enrofloxacin treatment level decreases as 2.7 logs. The downstream time points (24 and 48 hours) showed that IL-1 β gene expression significantly increased over time in the two antibiotic treated groups even surpassing levels from the infected-only group, which as described before started becoming up-regulated vs. the control not-infected group. At 24 h, of all groups gentamicin expressed the most indicating its strong effect in IL-1 β modulation. At 48 h, there was no significant difference between the two antibiotic-treated groups which reflect a converging effect. By the end of the experiment (6 days), gene expression of IL-1 β in the Roma-treated groups was lower than in infected-only group, although being higher than control. Interestingly, despite this reduction, both experimental groups remained higher in expression than the control non infected group by more than 2.5 log units.

TNFα gene expression dynamics under antibiotic treatments

Analysis of TNFα gene expression dynamics following APEC infection under different antibiotic treatments revealed clues to the complex interactions between host immunity and drug treatment (Fig. 7). At 3 hpi, two of the antibiotic-treated groups had a significant upregulation in TNFα RNA level compared with infected-only group; itself was with a high expresser (approx. 2.4 logs) when compared to uninfected control chickens. In the antibiotic groups, expression of TNFα at this early time point was 6.5 to 7.3 logs (Fig. 4 and 5), suggesting a strong early response. Remarkably, this was in contrast to IL-1 β; the infected-only group did not show an up-regulation at this time point.

With the development of infection, downregulation of TNFα mRNA was also observed in 2 different antibiotic groups at both 9 and 24 hours that fell below levels observed in infected-only mice. However, in the gentamicin group expression decreased to a 2 logs reduction at 9 hours, however interestingly it returned to that of control non-infected chickens from 24 hours onward. This upregulation indicated a brief effect of TNFα stimulation following gentamicin treatment. However, the group treated with enrofloxacin ended up with variable expression levels which did not return to base expression. It came in below the level observed in the infected group at 9 and 24 hours and was most down-regulated to around two logs at 24 hours, significantly this was its lowest level post treatment and infection for this experimental group. At the later time points 48 hours and 6 days, TNFα gene expression in the enrofloxacin-treated group approached levels of infected-only mice. This rebound at 48 hr (4.7

logs) in the enrofloxacin-treated group remained similar to infected-only birds over 6 days (3.7 logs).

IL-17 gene expression dynamics under antibiotic treatments

Following examination of the IL-17 gene expression kinetics in response to APEC infection and antibiotics administration, a unique pattern was developed with concentrations showing three peaks of massive induction at 3 h, 24 h, and 6 days post-infection/antibiotic treatment in gentamycin-treated birds (Fig. 8). By 3 hours, IL-17 had an unprecedented peak, ~12 logs above control non-infected chickens. This early peak was much greater than any of the expression levels observed in infected chickens. The peak had slightly declined, but was still significant compared with after 24 hours (approximately 9 logs). Surprisingly, the peak we found at 6 days post-infection was also 1 log lower, but it persisted at a high level (around 6 logs) with an opposite value than the one infected only +/- untreated group. A counterintuitive change was observed at 9 h post-infection after a down-regulation of IL-17 lower than the normal level with respect to the control non-infected chickens, which varied up to −0.2 logs. At 48 h, the expression was still high as compared with the control, but lower than the highs detected before and after this time point (24 h and six days). In the enrofloxacin-treated group, the IL-17 was followed similarly to that found in the APEC-infected group, but at 24 hpi surpassed this last in two logs of magnitude (4 logs vs.2 logs). Contrasting results were observed in the Gentamycin group, which behaved inversely with respect to both the other two groups (Enrofloxacin and APEC-infected only groups) under all the tested time points.

IL-6 gene expression after APEC infection with antibiotic treatments

In the gentamicin-treated group, IL-6 exhibited a remarkable surge at 3 hours, reaching almost 155 logs, doubling the production seen in APEC infection without antibiotics (Fig. 9). Enrofloxacin-treated birds also displayed a significant increase, reaching 46 logs and considered the lowest among all experimental groups. By 9 hours, both antibiotic-treated groups displayed a significant downregulation of IL-6. The gentamicin-treated group reached a level similar to healthy control birds (1 log), while the enrofloxacin-treated group dropped below the normal expression (0.8 log), both significantly lower than the infected-only group. At 24 hours, while the enrofloxacin-treated group returned to normal expression around 1 log, the gentamicin-treated group showed a slight increase (1.6 log), but remained lower than the infected-only birds.

A second wave of IL-6 upregulation occurred at 48 hours, with the enrofloxacin-treated group reaching the highest expression at 22 logs, while the gentamycin-treated group reached 12 log and later matched the expression detected in infected-only birds. At 6 days, all groups exhibited decreased expression compared to 48 hours, but IL-6 levels remained elevated in both antibiotic-treated groups. The gentamicin-treated group had the lowest expression at this final time point at 2.5 logs, which is not significantly different from the infected-only group, while the enrofloxacin-treated group showed the highest level at 5 logs.

IL-10 gene expression after APEC infection with antibiotic treatments

At 3 hours, IL-10 expression in both antibiotic-treated groups was below the normal level, ranging from 0.86 to 0.94 compared to healthy birds' expression (Fig. 10). In the gentamicin-treated group, a gradual increase was observed at 9, 24, and 48 hours (1.1, 1.6, and 3 logs, respectively). At the first two time points, IL-10 expression was lower than in the infected-only group, while at 48 hours no significant difference was detected. However, at 6 days, expression subsided below normal levels of healthy birds (0.66 log), scoring even the lowest among other experimental groups.

The IL-10 expression profile in the enrofloxacin-treated group exhibited a distinctive temporal pattern after 9 hours of infection and treatment. At this time point post-infection, IL-10 expression surged in the enrofloxacin-treated group, surpassing the other antibiotic-treated group at 3.6 logs but reaching a level comparable to the infected-only group. By 24 hours, a decrease ensued in the enrofloxacin-treated group, bringing IL-10 expression back to normal levels (1 log) of the healthy birds. At this juncture, the enrofloxacin-treated group exhibited the lowest IL-10 expression, significantly diverging from the infected-only group. Importantly, the difference between the enrofloxacin-treated and gentamicin-treated groups, although notable, did not achieve statistical significance. Interestingly, IL-10 expression in this group tremendously increased at 48 hours, reaching 13.5 logs, the highest among the experimental groups. By 6 days, expression decreased to levels close to the infected-only group (1.5 log), higher than the gentamycin-treated group. Intriguingly, this decline remained higher than the gentamicin-treated group, even though the latter group had dipped below the normal expression observed in healthy birds.

DISCUSSION

This current study investigated the in vivo efficacy of gentamicin and enrofloxacin against APEC challenged broiler chicks despite the in vitro resistant pattern. Bacterial hepatic colonization, mortality dynamics, and the modulation of different hepatic cytokine responses (IL-1β, TNF-α, IL-6, IL-10, IL-17) were also measured. Herein, we report a striking dichotomy between in vitro antibacterial antibiotic susceptibility testing and the ability to protect from APEC O78-induced infection of two common poultry antibiotics; gentamicin (susceptible in vitro) and enrofloxacin (resistant in vitro) while demonstrating profound immunomodulatory effects.

The tested APEC O78 isolate displayed the typical MDR profile known for current field strains, which are resistant to critically important antibiotics including colistin, fluoroquinolones (ciprofloxacin and enrofloxacin), tetracyclines, macrolides, penicillins and phenicols; only fosfomycin and gentamicin remain as susceptible agents based on (Wayne 2020). This pattern is in accordance with many other publications from different countries showing a resistant profile of APEC (Ievy et al. 2020; Thomrongsuwannakij et al. 2020; Hu et al. 2022).

Although fully resistant in vitro to enrofloxacin, daily treatment with this fluoroquinolone did not decrease and

even tended to increase cumulative mortality (44% vs 39.5% in the infected untreated animals; RR = 1.11, 95% CI: 0.78-1.59, P = 0.56). This concept, that antibiotic therapy in the setting of resistance can negatively impact the host, is likely due to the perturbation of the host microbiota and loss of colonization resistance, which may permit proliferation and growth of the pathogens (Caballero-Flores et al. 2023; Yuan et al. 2023). In addition, the early deaths found in the APEC-E inoculation group indicates that enrofloxacin treatment might have caused physical stress or changed initial host-pathogen interaction unfavorably. Even more prominently, at later times, the enrofloxacin-treated animals actually maintained slightly higher hepatic bacterial loads at 6 days (4.50 ± 0.15 $\log_{10}$ CFU/g) compared to gentamicin-treated birds (2.60 ± 0.31 $\log_{10}$ CFU/g; mean difference = -1.90, 95% CI: -2.73 to -1.07; Cohen's d = 3.56, representing a very large effect size). These results are consistent with previous reports showing that fluoroquinolones can induce overgrowth of extraintestinal pathogenic *E. coli* infections by resistant isolates, likely through selection for hypervirulent subpopulations or immunosuppression (Fuzi et al. 2020; Nasrollahian et al. 2024).

In striking contrast, gentamicin–the only other antibiotic to which the strain was fully susceptible and completely prevented mortality at a standard dose (0% mortality vs. 39.5% in APEC-only; RR = 0.00, 95% CI: 0.00-0.28; P < 0.001). This full protection also took place despite the fact that at all times gentamicin was only temporarily effective in preventing liver colonization (negative up to 24 h, then reappearance at a low level from 48 h). At 6 days, gentamicin-treated birds had significantly lower bacterial loads (2.60 ± 0.31 $\log_{10}$ CFU/g) compared to APEC-only (4.41 ± 0.18 $\log_{10}$ CFU/g; Cohen's d = 3.24) and APEC-E (4.50 ± 0.15 $\log_{10}$ CFU/g; Cohen's d = 3.56). Subsequent re-colonization at a low level, but with no mortality indicates that following the initial acute phase, birds are able to keep any remaining bacteria under control through an acquired immune response. This is supported by a review study about the bird's immune response to APEC as a primary pathogen which stated that after APEC infection in birds, an early strong pro-inflammatory response is achieved due to the host defense peptides and cellular innate immune response (Alber et al. 2021; Li et al. 2025). It's worth mentioning that incomplete bacterial clearance could be sufficient to hinder the mortality, however the bacterial colonization and disease recrudescence or even establishment of a carrier disease state could not be ruled out completely by which it is a very significant concern in a flock health status (Kobuszewska & Wysok 2024; Niu et al. 2024).

One of the most relevant and innovative results in our study is the strong immunomodulatory power of both antibiotics, really irrespective to their antibacterial action. Two-way ANOVA revealed significant group × time interactions for all cytokines in healthy birds (P < 0.001, η^2p ranging from 0.32 to 0.71), indicating large treatment effects. Gentamicin and enrofloxacin induced significant changes on hepatic cytokine transcription in healthy and infected birds, frequently with contrary effects depending on the cytokine analyzed and time point. In healthy non-challenged birds, the two antimicrobials downregulate the proinflammatory immune mediators (IL-1β, TNFα, IL-6 and IL-17) during the first 24 hours followed by subsequent a significant up-regulation response at 48 h and 6 days. This biphasic trend has been described with aminoglycosides and fluoroquinoles in mammals (Dalhoff & Shalit 2003; Labro 2000; Móritz et al. 2025; Wang et al. 2022), reflecting an early anti-inflammatory effect followed by rebound hyperesponsiveness. Of particular interest is the very strong peak of IL-6 at 48 h (12.2 ± 1.1 $\log_{10}$; Cohen's d = 4.87 vs. control) in healthy gentamicin-treated birds which may be indicative of the activation of alternate pathways (e.g, a direct stimulation of hepatocytes or Kupffer's cells). Similarly, gentamicin induced a massive IL-10 peak at 48 h (10.5 ± 0.9 $\log_{10}$; Cohen's d = 4.52 vs. control), while enrofloxacin produced more moderate but sustained elevations.

In acute APEC infection, the immunomodulatory effect increased further and was cytokine specific. Gentamicin drove an early massive and dramatic up-regulations of IL-1β (8.6 ± 0.7-fold at 3 h; vs. APEC-only: Cohen's d = 4.28), TNFα (6.5 ± 0.5-fold at 3 h), IL-6 (155.2 ± 12.4-fold at 3 h; vs. APEC-only: Cohen's d = 3.94) and in particular IL-17 extending 12.1 ± 1.0-fold at 3 h (vs. APEC-only: Cohen's d = 5.21). These changes far surpassing the response of those in untreated infected birds. This dramatic early cytokine storm contrasted paradoxically with 100% survival, indicating a protective rather than a detrimental role for a rapid and overwhelming innate response when concomitant bacterial killing is present. Relative "protective hyperinflammation" has been observed in *E. coli* sepsis of mice treated with appropriate antibiotics at the onset of disease (Osuchowski et al. 2006; Kim et al. 2024; Tosi et al. 2024). Furthermore, the usage of a proper antibiotic could modulate the host immune response in Gram-negative bacterial infections that is associated little if any additional inflammatory process with improved survival (Gross et al. 2024; Aloni-Grinstein et al. 2025).

Although enrofloxacin did not possess bactericidal effects, it stimulated early proinflammatory response (IL-6: 46.1 ± 5.2-fold at 3 h; significantly lower than gentamicin: Cohen's d = 5.18) however followed by times of cytokine downregulation especially for TNFα and IL-6 at 9–24 h. At 9 h, both antibiotic-treated groups had IL-6 expression significantly lower than APEC-only (P < 0.001). This immune response fluctuation may account for increased bacterial burden and birds' mortality that seen in enrofloxacin-treated infected chickens. Notably, at 48 h, enrofloxacin-treated birds exhibited a second wave of IL-6 (22.4 ± 2.1-fold), significantly exceeding both gentamicin-treated and APEC-only groups (Cohen's d = 2.59 and 3.17, respectively).

The regulatory cytokine IL-10 had a complex kinetics. Gentamicin induced only a moderate amount of IL-10, with a peak of 3.0 ± 0.4-fold at 48 h, while the enrofloxacin elicited a massive late-phase surge (13.5 ± 1.2-fold at 48 h; vs. APEC-G: Cohen's d = 5.47; vs. APEC-only: Cohen's d = 4.95). A detrimental role for enrofloxacin in this model is compatible with the association of an excessive IL-10 response with impaired bacterial clearing and increased mortality from multiple Gram-negative infections (van der Poll & Opal 2008; Gong et al. 2024; Brandquist & Kielian 2025) thereby providing a potential mechanistic explanation.

Taken together, these data strongly suggest that the clinical outcome of APEC infection in poultry treated with antimicrobials is not only determined by direct bacterial killing mechanism but by the net immunomodulatory effect of the antimicrobials on the host response. The significant group × time interactions observed for all cytokines (η^2p = 0.74-0.80) demonstrate that antibiotic treatment fundamentally alters the temporal dynamics of the immune response to infection.

One of the important limitations of the current study is the limitation of that cytokine expression to the livers and only restricted to mRNA over expression. Despite that the hepatic tissues are the major sites of bacterial colonization, acute phase response, and Kupffer cell activation during systemic APEC infection, this does not completely reflect the complete scenarios of the systemic immune response. In poultry, APEC infection affects different systemic organs and not limited to the livers (Joseph et al. 2023; Logue et al. 2025; Zaki et al. 2025). Each affected organ may exhibit distinct cytokine profiling and immune response reflecting tissue-specific host–pathogen interactions and local immune regulation (Sadeyen et al. 2014; Wickramasuriya et al. 2022; Chen et al. 2025). Therefore, the current hepatic cytokine expression cannot be extrapolated to reflect the entire systemic inflammation and immune responses at primary sites of pathogen entry. Furthermore, because mRNA data do not always relate to functional production of protein or bioactivity, post-transcriptional processes may greatly affect cytokine availability. Also, the lack of data from circulating cytokine quantification, immune cell phenotyping, histopathological scoring of immune cells, or functional assays (e.g, phagocytosis or bacterial elimination) will prevent us from making any mechanistic conclusions regarding the proposed immunomodulatory effects of the antibiotics.

This study only included one APEC O78 isolate, spanned 6 days, and only focused on measuring cytokine responses at the level of mRNA and only in the liver. The study also did not assess long-term immune responses, adaptive immunity or changes to the immune-modulating microbiome, any of which could account for the reported immune-modulating responses. Future studies should include other APEC serotypes, assess cytokine responses at the protein level, measure immune responses in multiple organs, and include the microbiome. Conducting these studies in the field and including immune profiling in the antibiotic selection process may provide insights on the effects of antibiotics on host immunity and improve the strategies available to treat infections caused by APEC.

Conclusion

In conclusion, this study provides compelling evidence that gentamicin remains a highly efficient antibiotic in poultry against studied multi-resistant APEC O78 (0% mortality, large effect sizes for bacterial reduction and immune modulation), while enrofloxacin, despite being one of the most widely used antibiotics in poultry worldwide may exhibit a negative impact on the health status of birds when resistance is present (44% mortality, dysregulated cytokine responses with very large effect sizes). Moreover, both antibiotics have powerful, cytokine-specific immunomodulatory effects that can dramatically alter disease outcome independent of direct antibacterial activity, as evidenced by the significant group × time interactions and large Cohen's d values for key cytokine comparisons. These findings highlight the urgent need to incorporate immune response profiling into antibiotic efficacy studies for veterinary pathogens. It is important to be noted that the current results are derived from a controlled experimental infection model and mechanistic hypotheses and remain to be validated. Therefore, these findings should be interpreted within this context.

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Ethics Statement: The study was read and approved by Jordan University of Science and Technology Animal Care and Use Committee under a protocol number 38/2022.

Author's Contribution: WH and MK conceptualized the study and designed the study. TA collected the data and executed the experiments and the statistics. WH wrote the original draft. MK supervised the study, reviewed and edited the manuscript. All authors reviewed and approved the manuscript

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