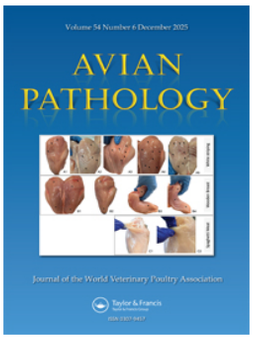

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Comparative pathogenesis of aerosol-induced colibacillosis reveals greater susceptibility in broiler chicks compared to layer chicks, with systemic clearance of bacteria but persistence in the bursa of Fabricius

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ABSTRACT

Avian colibacillosis presents significant challenges to the poultry industry by adversely affecting bird health and productivity and raising global public health concerns. While numerous studies on colibacillosis in broiler and layer chickens have been reported, a comparative investigation on the disease progression following respiratory infection in these two chicken types under identical conditions is lacking. This study aimed to compare the clinical, pathological, and microbiological features in broilers and layers experimentally infected with *ilux2*-marked *E. coli* PA14/17480/5-ovary strain (*ilux2*-APEC). Sixty-four 2-week-old specific pathogen-free chicks (32 broilers, 32 layers) were divided into four groups ($n = 16$), with control and infected groups for each type. Birds were necropsied at 3, 7, 10, and 14 days post-infection (dpi). In infected groups, spleen-to-body weight ratios were significantly higher compared to their respective controls. Mean maximum clinical scores, macroscopic, and microscopic lesions were significantly higher in infected broilers than in infected layers. Infected broilers showed a higher incidence of *ilux*2-APEC re-isolation from respiratory tissues compared to infected layers. Additionally, *ilux*2-APEC was re-isolated from blood, femoral head, heart, liver, and spleen exclusively in infected broilers. Unexpectedly, bioluminescence imaging identified bacterial colonization in the bursa of Fabricius in both infected groups, persisting up to 14 dpi. Immunohistochemistry detected *Escherichia coli* in trachea, lung, air sac, heart, liver spleen, and bursa of Fabricius, with higher detection rates in infected broilers. These findings highlight the increased susceptibility of broilers to APEC infection, suggesting a need for targeted disease control measures and potential genetic selection for improved disease resistance in broilers.

RESEARCH HIGHLIGHTS

- Broiler birds as compared to layers are more susceptible to avian colibacillosis.
- An aerosol challenge using an *ilux2*-APEC effectively evaluates disease pathogenesis.
- The *ilux2*-APEC persistently colonized the bursa of Fabricius after aerosol exposure.

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Introduction

Avian pathogenic *Escherichia coli* (APEC), a subgroup of extraintestinal pathogenic *E. coli*, is responsible for a range of extraintestinal infections in birds, collectively known as avian colibacillosis. The disease is a multifaceted syndrome that affects various sectors of poultry production, including broiler and egg-laying chickens. It can appear in many forms, such as airsacculitis, pneumonia, pericarditis, perihepatitis, peritonitis, salpingitis, osteomyelitis/synovitis, omphalitis, and cellulitis (Nolan *et al.*, 2020). APEC infections result in significant economic losses for the poultry industry due to decreased productivity in affected birds, increased morbidity and mortality rates, high condemnation of infected carcasses in slaughterhouses, and high costs associated with prevention and treatment (Davis *et al.*, 2018; Nolan *et al.*, 2020). The bacteria can also be transmitted to chicks during the hatching process, resulting in high mortality rates in newly hatched chicks or yolk sac infections (Petersen *et al.*, 2006; Roberts *et al.*, 2011; Poulsen *et al.*, 2017). APEC can also negatively impact egg production and quality in laying hens by causing salpingitis, which may lead to

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vertical transmission to the eggshell, egg white, and yolk (Abdelhamid *et al.*, 2024). Notably, antibiotic-resistant strains of *E. coli* have emerged worldwide, and ability to induce a broad protection by the existing vaccines is a great concern (Paudel *et al.*, 2024). From a public health point of view, comparative genomics showed that some APEC isolates appear closely related to human extra-intestinal *E. coli* strains, including uropathogenic *E. coli*, septicaemia-causing *E. coli*, and neonatal meningitis-causing *E. coli*, supporting the potential zoonotic risk associated with APEC (Mellata, 2013; Jeong *et al.*, 2021; Meena *et al.*, 2021). Avian *E. coli* contaminated chicken meat and egg products are thought to be a potential vehicle for infection by extra-intestinal *E. coli* in humans (Mitchell *et al.*, 2015; Zhuge *et al.*, 2019; Sharan *et al.*, 2023).

Genetic selection of poultry resulted in two distinct chicken breeds, broiler-type and layer-type, which differ significantly in weight gain, behaviours, physiological parameters, and immune responses (Koenen *et al.*, 2002; Yan *et al.*, 2021). The demand for poultry meat has increased substantially in recent years and is expected to continue rising due to human population growth and greater *per capita* consumption (OECD/FAO, 2023). In response, there is a pressing need to boost growth rates and meat yields through commercial genetic and genomic selection of broiler chickens. In broilers, performance was prioritized at the expense of immune function, making birds more susceptible to disease compared to their ancient breeds (Borodin *et al.*, 2020; Zou *et al.*, 2020). Studies have shown that fast-growing birds often have a compromised adaptive immune response, which increases their risk for infectious diseases (Cheema *et al.*, 2003).

Despite extensive research on the pathogenesis of colibacillosis in broiler and layer chickens (Kromann & Jensen, 2022), it is quite challenging to compare existing studies, as the conditions in most reports differ with respect to APEC serotypes, doses administered, routes of inoculation, chicken breeds, age, etc. So far, no comparison of the pathogenesis of colibacillosis in SPF broiler-type and SPF layer-type chicks has been conducted in an identical experimental conditions. Therefore, the aim of the present study was to compare the pathogenesis of APEC infection in SPF broiler-type and SPF layer-type chicks of the same age under comparable conditions, utilizing the previously developed *ilux*2-*E. coli* PA14/17480/5-ovary bioluminescent strain for aerosol infection. Progression of the disease and pattern of bacterial colonization in the two bird types were evaluated. The findings are expected to highlight the need for targeted infection control strategies and effective management against this pathogen in more susceptible bird type, ultimately leading to improved poultry welfare and more sustainability in poultry production systems.

Materials and methods

Bacterial isolate and preparation of inoculum

To easily track the bacteria and differentiate experimental vs. native *E. coli* strains, the *ilux2-E. coli* PA14/17480/5-ovary (*ilux2*-APEC) strain was used for infection. The *ilux2*-APEC used in this study was established by chromosomal insertion of the *ilux2* operon (Gregor, 2022) into the *E. coli* PA14/17480/5-ovary as described recently (Abdelhamid *et al.*, 2024). The *E. coli* PA14/17480/5-ovary (parent strain) isolate was collected from the ovary of a layer chicken with colibacillosis (Rezaee *et al.*, 2021). The *ilux2*-APEC, similar to its parent strain, met all the criteria to be classified as APEC as based on *in vivo* characteristics (Abdelhamid *et al.*, 2024, 2025). For infection, the *ilux2-E. coli* culture was washed and resuspended in phosphate buffered saline (PBS) to prepare the inoculum. The final bacterial concentration was quantified by direct plating of 100 µl of serial dilutions on LB agar plates and incubating at 37°C for 24 h, to determine number of colony-forming units (CFU)/ml (Paudel *et al.*, 2023). The bioluminescence emission of the *ilux2*-APEC isolate was visualized using an *in vivo* imaging system (IVIS) instrument with an advanced camera capable of capturing the bioluminescent signal (Lumina LT, PerkinElmer, Rodgau, Germany).

Experimental design, bird housing, and sampling

The animal trial was approved by the institutional ethics committee and the national authority according to § 8ff of the law for animal experiments, Tierversuchsgesetz-TVG (licence number: GZ 2023-0.849.898).

Two different SPF chicken types were used in this study to compare the infection dynamics of APEC: SPF broiler-type (Royal GD, Deventer, Netherlands) and SPF layer-type (VALO Biomedia GmbH, Osterholz-Scharmbeck, Germany). The SPF broiler and layer eggs were incubated and hatched in our facility at the Clinic for Poultry and Fish Medicine, University of Veterinary Medicine Vienna, Austria. After hatching, 32 birds from each bird type were randomly divided into an experimentally infected group and a negative control group, with 16 birds each (Table 1). One-day-old chicks were neck-tagged for identification, and birds of each group were housed in a 2×1.5 metre pen within a negatively pressured room, on deep litter with *ad libitum* access to the same feed and water to ensure consistency across all groups. At 2 weeks of age, SPF broiler and SPF layer chicks of the challenged

Table 1. Experimental design of animal study.

Groups	Number of birds	Aerosol inoculation (2 weeks of life)	Necropsy and sampling			
			3 dpi[a]	7 dpi	10 dpi	14 dpi
Infected broiler	16	*ilux2-E. coli* PA14/17480/5-ovary	4[b]	4	4	2[c]
Non-infected brolier	16	PBS	4	4	4	4
Infected layer	16	*ilux2-E. coli* PA14/17480/5-ovary	4	4	4	4
Non-infected layer	16	PBS	4	4	4	4

[a]dpi: days post-infection.
[b]Number of birds killed at each time-point.
[c]Two chicks were euthanized at 4 dpi due to neurological signs.

groups were infected with 5×10^8 CFU/ml *ilux2-E. coli* PA14/17480/5-ovary isolate via the aerosol route. Sterile PBS was administered in negative control groups. For aerosolization, NebulAir$^{+®}$ (Flaem, Martino della Battaglia, Italy) was used to generate aerosol droplets < 5 µm (81.5%). Sixteen birds from each group, either infected or non-infected, were placed in a plastic box (L = 57 cm, W = 39 cm, H = 28 cm). The nozzle of the nebulizer was then inserted and fixed into a pre-drilled hole fitting with the nozzle contour. A volume of 16 ml of either the bacterial inoculum for each of the infected groups or PBS for each of the negative control groups was aerosolized inside the box through the nebulizer (Figure 1). Following aerosol exposure over a total duration of 25 min, birds were taken out of the aerosol boxes and monitored daily for clinical signs according to the scoring scheme presented in Supplementary Table S1. For necropsy and sampling, birds from each group were sequentially euthanized at 3, 7, 10, and 14 days post-infection (dpi), and macroscopic lesions were scored during necropsy using a scoring system (Abdelhamid *et al.*, 2024).

Organ-to-bodyweight ratio

During necropsy, the total bodyweight and the weights of liver and spleen of each bird were recorded, and the organ-to-bodyweight ratio was calculated as (weight of organ/bodyweight) × 100.

Bioluminescence imaging

All birds from the infected groups were imaged for detection of the bioluminescent signals emitted by the *ilux2*-APEC following a previously published protocol using an *in vivo* imaging system instrument (IVIS Lumina LT, PerkinElmer, Rodgau, Germany) with a binning of 16 (large) and a f/stop of 1 (Abdelhamid *et al.*, 2024). Briefly, each killed bird was deskinned, and the whole body was imaged from both dorsal and ventral sides. Afterwards, necropsy was performed, and internal organs, including trachea, lung, heart, liver, spleen, and brain, were imaged separately. The bioluminescent signal was reported as total flux (radiance: photons (p)/second (sec)/cm^2/steradian (sr)), where steradian represents the photons emitted from a unit solid angle of a sphere.

Bacteriology

For bacteria re-isolation, the trachea, lung, air sac, heart, liver, spleen, and brain of necropsied birds were plated on LB agar plates. Two cotton swab smears were collected from the inside surfaces of the aerosol plastic boxes immediately following the aerosol infection and were similarly cultured on LB agar. Additionally, the femoral head and bursa of Fabricius were included for bacteriological investigations from the birds euthanized at 7, 10, and 14 dpi. The plates

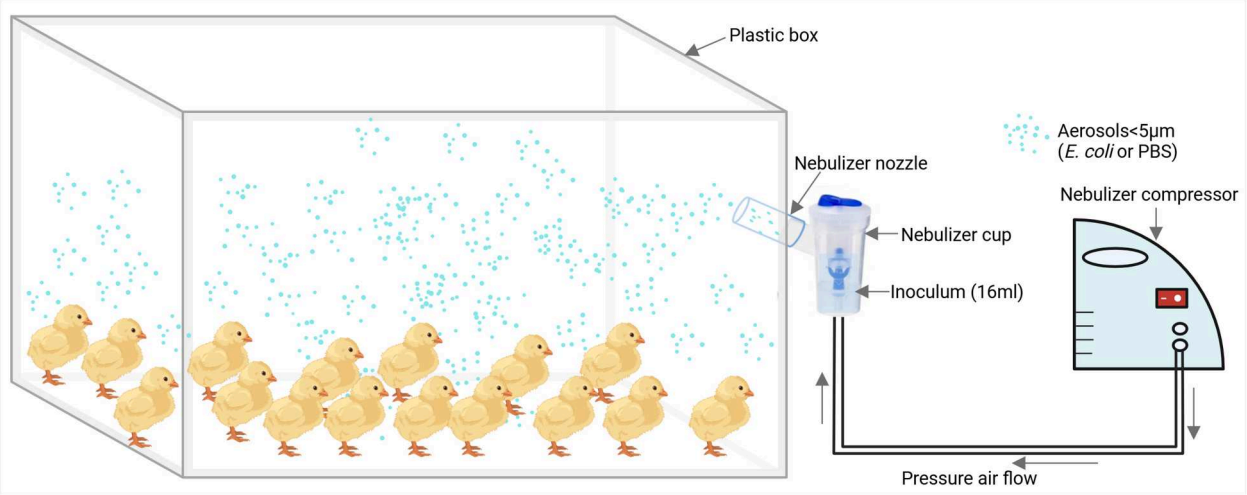

Figure 1. Schematic representation of the aerosol exposure system designed for infecting birds with nebulized aerosols. The figure was created with the aid of BioRender.com.

were incubated aerobically at 37°C overnight and were then examined under IVIS for detection of bioluminescent colonies.

Histopathology

Tissue samples from the trachea, lung, air sac, liver, spleen, brain, and bursa of Fabricius were collected during necropsy. Samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned into 5 µm slices, and stained with haematoxylin and eosin for microscopic examinations. The microscopic lesions in affected organs were scored and categorized by lesion score (LS) 0–2 using an previously established histopathological scoring scheme (Paudel *et al.*, 2021, 2023) with some modifications (Supplementary Table S2).

Immunohistochemistry (IHC)

For *E. coli* detection in the trachea, lung, air sacs, liver, spleen, brain, and bursa of Fabricius, paraffin-embedded tissue sections were processed for IHC using an anti-*E. coli* LPS antibody (2D7/1, Abcam, Cambridge, UK) following a previously published protocol (Abdelhamid *et al.*, 2021). The VECTASTAIN® ABC kit and DAB substrate kit (Vector Laboratories, Newark, CA, USA) were used for signal visualization. Counterstaining was done with Mayer's haematoxylin (Merck KGaA, Daemstadt, Germany), and the colour reaction was observed under the microscope.

Statistical analysis

GraphPad Prism 9.2.0 software (GraphPad Software Inc., San Diego, CA) was used to perform statistical analysis and create graphs. Data of clinical score, organ-to-bodyweight ratio, and macroscopical and histological lesion scores were analysed using an independent t-test if the data were normally distributed, and a Mann–Whitney test as a nonparametric approach when data did not comply with normal distribution. The data were presented as mean ± SEM, and statistical significance was considered at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Results

Clinical signs

Clinical signs varied among APEC-infected groups, with the mean maximum disease score (the mean of the maximum clinical score for each bird per group) being significantly higher in the infected broiler group than in the infected layer group ($P < 0.05$, Figure 2). In the group of infected broiler birds, out of 16 birds, eight showed mild clinical signs (score 1), two birds showed moderate clinical signs (score 2), and two birds had to be euthanized at 4 dpi (prior to the scheduled time-point at 7 dpi) due to severe neurological signs. These signs included distressing behaviours in the form of tucking the head between the legs and moving rapidly backwards. In contrast, only six out of 16 infected layer birds showed clinical score 1. Both non-infected groups of broilers and layers remained clinically healthy throughout the experiment.

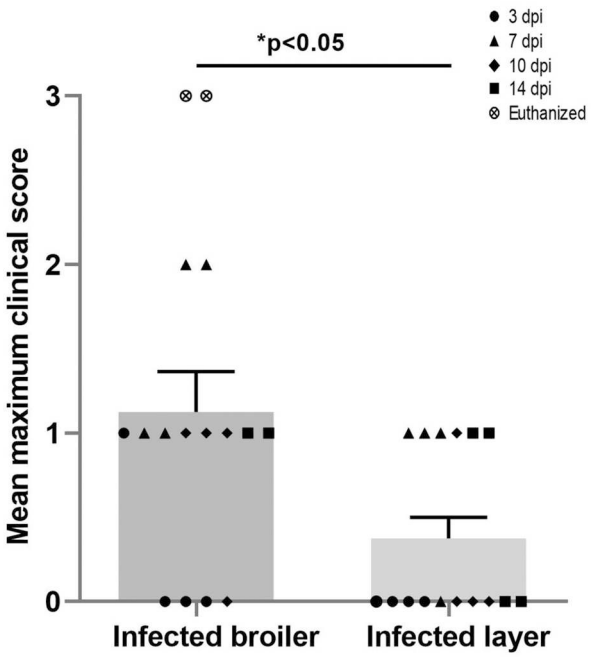

Figure 2. Mean maximum clinical disease score for infected birds from each group. Clinical signs were scored as: 0 = no signs, 1 = slightly weak, dropping wings, with depressed; 2 = weak, with ruffled feathers, reluctant to move, apathy; 3 = bird unable to stand with severe neurological signs. Each symbol represents the maximum clinical sign score of a single bird over a period starting from 1 day post-infection until the time of necropsy. Error bars represent the ±SEM. $^{*}P < 0.05$ denotes statistically significant differences between infected broiler and layer birds.

Pathological lesions

Birds from the control groups did not exhibit any macroscopic lesions during *postmortem* examination (Figure 3(A)). In contrast, APEC-infected birds showed lesions of varying severity which were characterized as serofibrinous exudate to caseous exudate of various quantities in the lung, air sacs, heart, liver, and peritoneum (Figure 3(B,C)). A comparison of pathologic lesion scoring of the lung, air sacs, heart, liver, spleen, and peritoneum in chicks of the infected groups demonstrated significantly higher mean lesion score in the APEC-infected broiler group than in the layer group (Figure 3(D)).

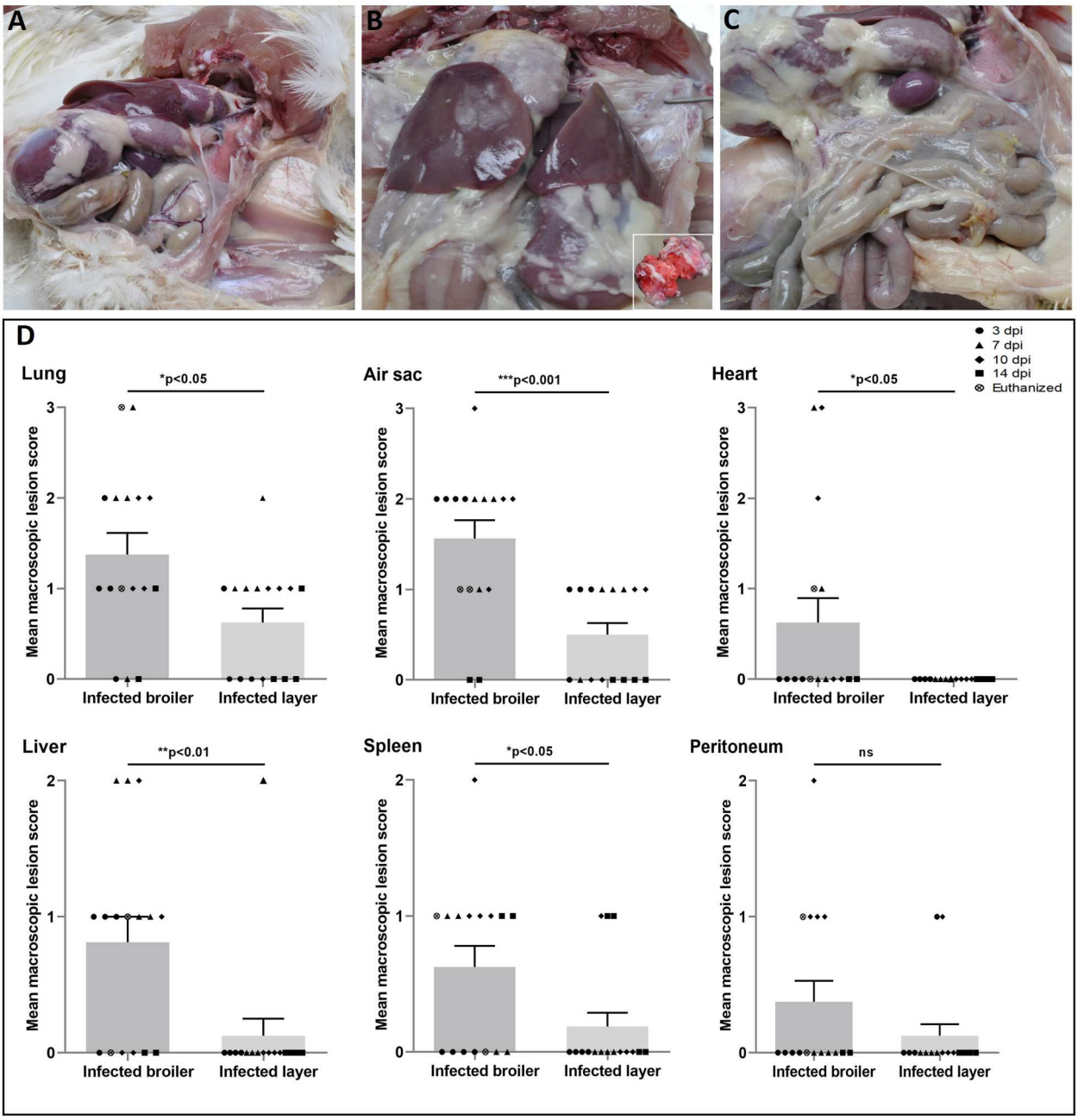

Figure 3. Macroscopic lesion scores. (A) No lesions were observed in groups exposed to aerosolized PBS; (B) Different amounts of fibrin deposits found on the heart, air sac, liver, and lung (inset) of APEC aerosolized broiler birds; (C) Thickening of the peritoneum accompanied by fibrin deposits on its surface in APEC aerosolized broiler birds; (D) Average macroscopic lesion scores for the lung, air sac, heart, liver, spleen, and peritoneum in APEC aerosolized groups. Each symbol represents the lesion score for an individual bird within each group ($n = 16$). Error bars represent the ±SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ denote statistically significant differences between infected broiler and layer groups, while ns indicates no significant differences.

Organ-to-bodyweight ratio

No significant difference in the liver-to-bodyweight ratio was observed in APEC-infected broiler and layer groups as compared to their respective negative controls (Figure 4(A)). In contrast, the spleen-to-body-weight ratio was significantly higher in both infected groups relative to their non-infected control groups. Specifically, the infected broilers exhibited a highly significant increase ($P < 0.0001$), while the infected layers showed a significant increase ($P < 0.01$) (Figure 4(B)).

Bioluminescence imaging

Bioluminescence imaging of whole deskinned bodies revealed a detectable signal in the caudal aspect over the cloaca when imaged from the dorsal side in one bird from the broiler infected group that was euthanized at 4 dpi. Further imaging of internal organs *postmortem* identified the origin of this signal specifically from the bursa of Fabricius (Figure 5(A)). Additionally, *postmortem* bioluminescence imaging revealed a signal from the femoral head of another bird necropsied at 3 dpi (Figure 5(B)) and

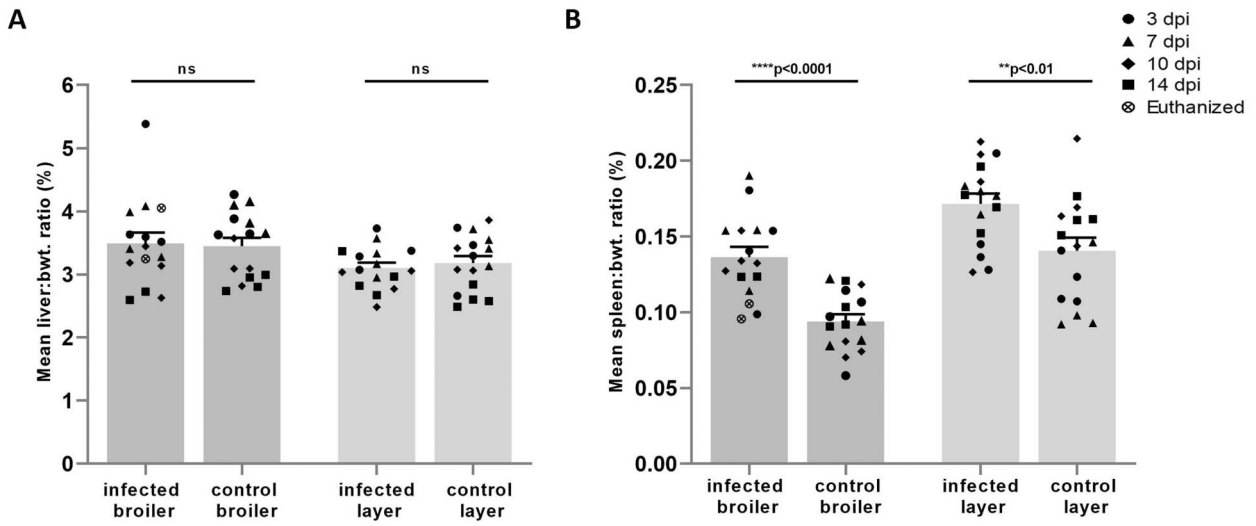

Figure 4. Organ-to-bodyweight (bwt) ratios for liver (A) and spleen (B). Each symbol represents the organ-to-bodyweight ratio of an individual bird within each group ($n = 16$). Error bars represent ±SEM. $^{**}P < 0.01$ and $^{****}P < 0.0001$ denote statistically significant differences between each infected group and its respective negative control, while ns indicates no significant differences.

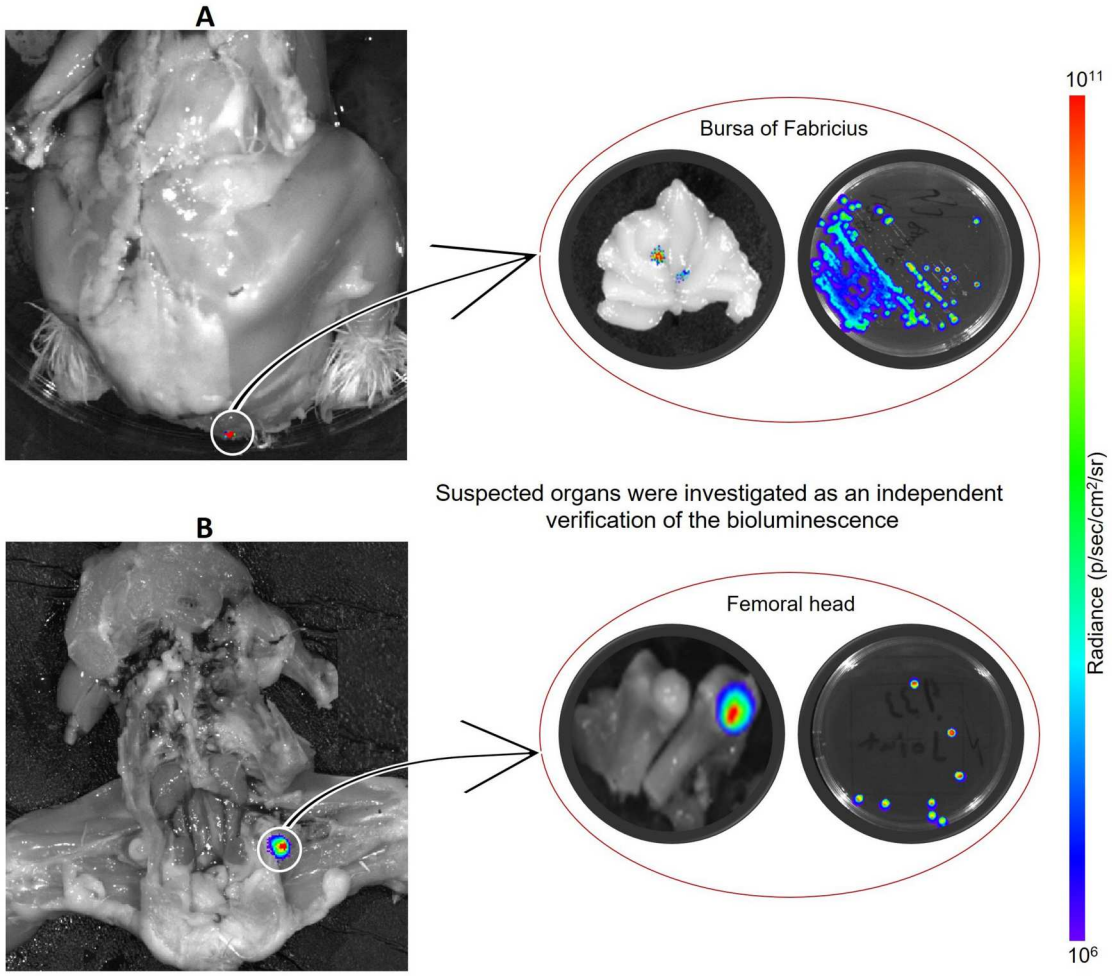

Figure 5. *Postmortem* bioluminescence imaging of *ilux2-E. coli* PA14/17480/5-ovary in infected chicks showed bioluminescence at two specific locations: the bursa of Fabricius (A), and the femoral head (B), with successful isolation of *ilux*2-APEC on LB agar plates from both locations.

from the lung of the euthanized bird at 4 dpi. Surprisingly, none of the target organs known for APEC colonization and lesions (such as trachea, air sac, heart, liver, and spleen) revealed any bioluminescence. However, bioluminescence signal emissions from the bursa of Fabricius were observed in four out of 11 infected broiler birds and one out of 12 infected layer birds.

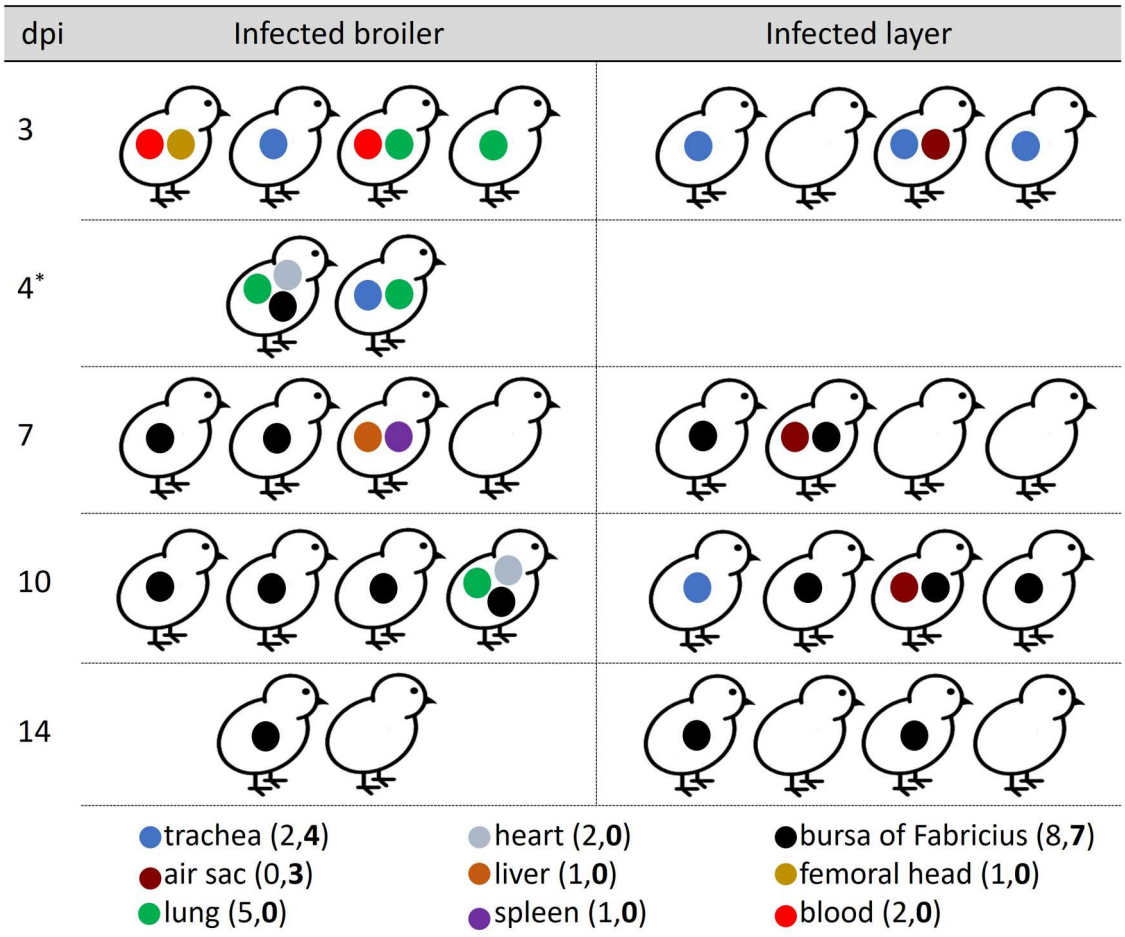

Figure 6. Re-isolation of *ilux*2-*E. coli* PA14/17480/5-ovary from various organs of birds in infected broiler and layer groups at different days post-infection (dpi). Different colours represent the different organs from which the bacteria were re-isolated. The total number of birds positive for bacterial re-isolation in each organ is indicated in parentheses, with regular numbers for the broiler group and bold numbers for the layer group. *Two birds were euthanized at 4 dpi before the scheduled necropsy due to severe neurological signs. The bursa of Fabricius was assessed for *ilux*2-APEC re-isolation in necropsied birds at 7, 10 and 14 dpi, following the bioluminescence detection in a bird that was euthanized at 4 dpi.

Bacterial re-isolation

The cumulative number of organs that tested positive for *ilux*2-APEC re-isolation was higher in the infected broilers ($n = 22$) than in the infected layers ($n = 14$) (Figure 6). Specifically, samples from the lung, liver, heart, spleen, blood, and femoral head tested positive only in chicks from the infected broiler group. Following bioluminescent detection of *ilux*2-APEC in the bursa of Fabricius of the bird euthanized at 4 dpi, the organ was included in the sampling scheme at 7, 10, and 14 dpi with successful re-isolations from the majority of infected birds (broiler, $n = 8$ or layer, $n = 7$). While this persisted until termination of the study at 14 dpi, all other respiratory and systemic organs tested negative for re-isolation. Furthermore, among the positive bursa of Fabricius samples, four from the infected broilers and five from the infected layers showed pure *ilux*2-APEC colonies, while the remaining showed a mix of bioluminescent and non-bioluminescent colonies. In the negative control groups six broilers and five layers showed growth of non-bioluminescent colonies on agar plates. Randomly selected non-bioluminescent bacterial colonies ($n = 6$), including samples from two infected birds and one negative control bird from each group, were identified as *Klebsiella pneumoniae* or *Enterobacter cloacae* using MALDI-TOF Mass Spectrometry. No bacteria were detected in any other organs from the birds in the negative control groups.

All swabs from the inner surfaces of the boxes used for the *ilux*2-APEC aerosol infection in both the broiler and layer infected groups tested positive for *ilux*2-APEC re-isolation. All swabs from the boxes used for PBS aerosolization in the negative control groups tested negative.

Histopathological lesion scores

Histopathological lesion scores in the trachea, lung, air sac, liver, spleen, and heart, together with representative pictures of negative controls with no lesions and those with the most severe changes observed in infected birds are shown in Figure 7.

Trachea (Figure 7, row A): in the trachea of infected broiler and layer chicks, only LS1 was observed, with higher prevalence in broiler birds. Lesions involved deciliation with irregular sloughing of tracheal epithelium, occasionally attached to bacterial bacilli.

Lung (Figure 7, row B): the mean lung lesion score was significantly higher in the infected broiler group than in the infected layer group. None of the infected layer birds had LS2, while five infected broilers showed LS2, which was characterized by the infiltration of mononuclear cells and heterophilic cells along with haemorrhage into lung tissue and parabronchial lumen.

Air sac (Figure 7, row C): differences in the lesion severity of the air sac were similar to those in the lung, with the mean air sac lesion score significantly higher in the infected broiler compared to the infected layer group. Air sacs with the most severe changes (LS2) were only seen in four infected broiler birds, but not in any infected layer birds. In the severely affected air sac, the lesion comprised thickening of air sac membranes with oedema and severe infiltration of heterophils and mononuclear cells, which in some birds progressed to caseous necrosis.

Heart (Figure 7, row D): none of the birds in the infected layer group showed heart lesions. In the infected broiler group, five birds showed mononuclear cell infiltration with heterophils in the epicardium (LS1), and one chick showed epicardial thickening with fibrinous exudate and heterophilic infiltration extending to the myocardium (LS2).

Liver (Figure 7, row E): LS1 was recorded only in the liver of infected birds, with higher prevalence in broiler birds ($n = 10$) than in layer birds ($n = 1$). Likewise, the mean liver lesion score was significantly higher in the infected broiler group compared to the infected layer group. Lesions involved thickening of the hepatic capsule with fibrinous exudate and heterophilic infiltration.

Spleen (Figure 7, row F): heterophilic infiltration was observed in the spleens of infected birds as compared to uninfected controls, with lesion scores ranging from LS1 to LS2. Variations in spleen lesion scores were observed between the infected groups, yet statistically significant difference was not detected.

Bursa of Fabricius: lesions in birds of both infected groups were restricted to focal desquamations of bursal epithelium.

Immunohistochemistry

The number of positive organs in each infected group is presented in Table 2. The number of *E. coli*-positive organs identified by IHC was higher in infected broilers as compared to the infected layers. *E. coli* was detected in respiratory and/or systemic organs in 93.75% of the infected broiler birds, while it was not detected in the air sac or liver of any of the infected layer birds. In the trachea, *E. coli* was detected as an individual *E. coli* bacillus attached to the desquamated tracheal epithelium (Figure 8(A)), whereas in the lung and air sacs, *E. coli* was detected in areas of inflammation and necrosis (Figure 8(B,C)). In the heart, it was present in the epicardium (Figure 8(D)). In the liver and spleen, *E. coli* was detected in tissue parenchyma (Figure 8(E,F)). Interestingly, a high number of birds in both infected groups were positive for *E. coli* antigen in the bursa of Fabricius. *E coli* was found mostly in bursal lumen, follicle-associated epithelium (FAE), and rarely as individual *E. coli* bacilli in the medulla of bursal follicle (Figure 8(G–I)). No *E. coli* was detected in any of the organs from the negative control groups.

Discussion

The main aim of the current study was to compare the susceptibility of SPF broiler-type and SPF layer-type chicks of the same age, raised under comparable conditions, and exposed to the same APEC infection. By exploring the differences in infection rates, clinical signs, macroscopic and microscopic lesions, and the extent of systemic invasion between these two chicken types, this study sought to contribute valuable insights into avian health management as no such study has been performed previously under identical experimental settings.

Different routes of inoculation, including respiratory, intraperitoneal, intravenous, intrauterine, and subcutaneous, have been used for inducing colibacillosis in chickens. Since APEC is mainly transmitted through the inhalation of contaminated particles, the aerosol infection model is proposed to be the optimal method for reproducing a natural infection appearing under field conditions (Paudel *et al.*, 2021; Saliha *et al.*, 2022; Paudel *et al.*, 2023; Rychlik *et al.*, 2023). Administration of *E. coli* via aerosol closely mimics a natural infection route different to direct invasive delivery of *E. coli* culture into target organs. Hence, birds in the current experiment were exposed to aerosolization either with APEC for infected groups or with PBS for the negative control groups. A nebulizer was used in the current study for generating aerosol particles of <5 μm in size. This size was shown to be efficient for the deposition of aerosolized particles carrying APEC bacteria deeply in the respiratory tract, inducing lesions pathognomonic for APEC infection (Paudel *et al.*, 2021). Moreover, a nebulizer was shown to be more efficient than an atomizer for producing avian colibacillosis at young ages (Saliha *et al.*, 2022). Despite birds in both infected groups being exposed to the same APEC strain, same dose, and same concentration under the same conditions, birds in the infected layer group showed significantly

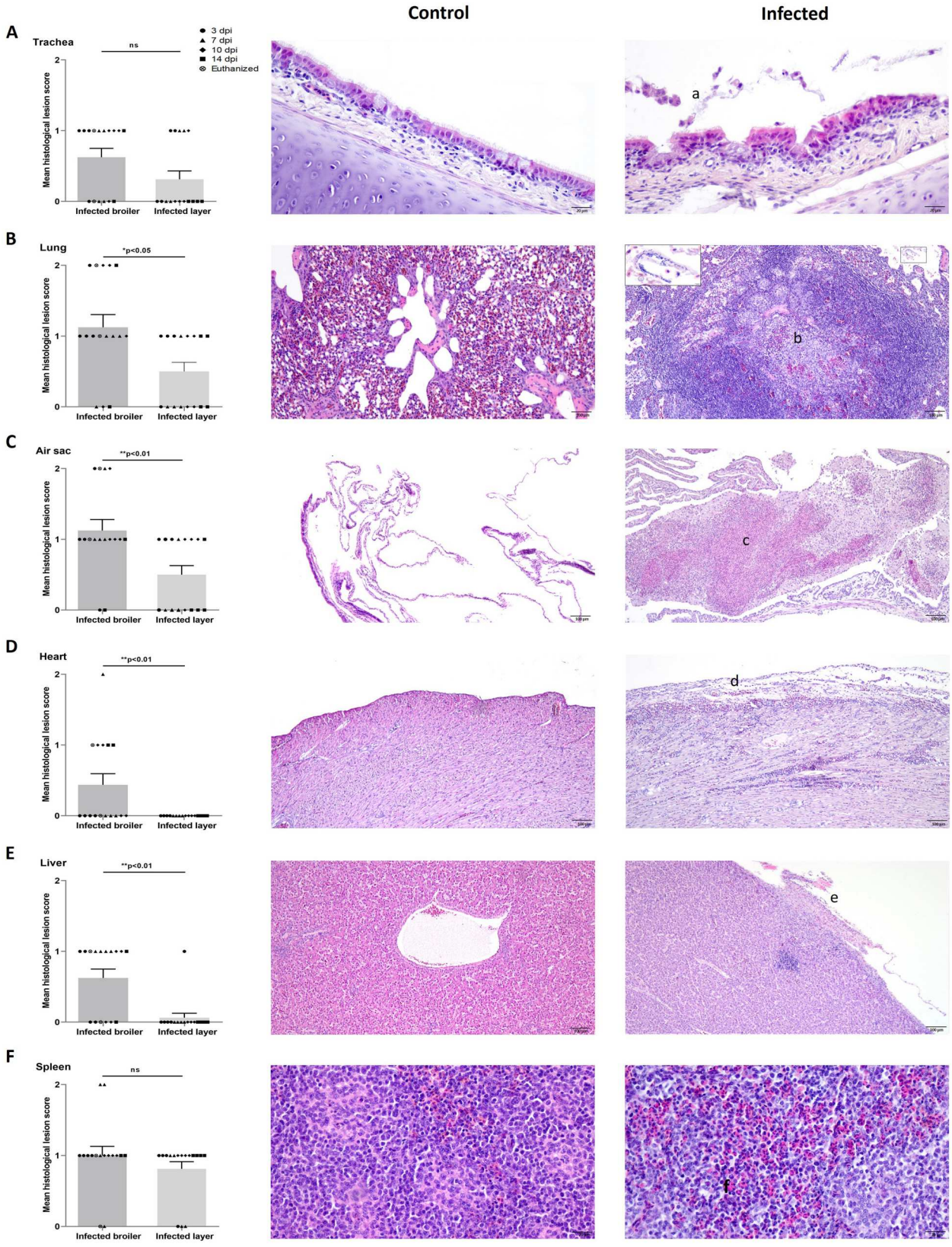

Figure 7. Average lesion scores and histopathological lesions for trachea (A), lung (B), air sac (C), heart (D), liver (E), and spleen (F). The representative histological images show normal structures with no lesions in the non-infected group, alongside the highest lesion scores observed in APEC-infected birds from both broiler and layer groups. Desquamated tracheal epithelial cells alongside attached *E. coli* bacteria (a). Severe infiltration of mononuclear and heterophil cells in the parabronchi and lung tissue, with the boxed area magnified to reveal sloughed epithelium colonized by *E. coli* bacteria (inset, bar 20 µm) (b). Air sac thickened by severe inflammation marked by many degranulating heterophils, bacteria, and fibrin (c). Thickening of epicardium with fibrinous exudate and heterophilic infiltration extending to the myocardium (d). Localized layer of fibrino-suppurative exudate overlying the capsule of the liver (e). Marked heterophilic infiltration (f). Error bars represent the ±SEM. *$P < 0.05$ and **$P < 0.01$ denote statistically significant differences between infected broiler and layer birds, while ns indicates no significant differences.

Table 2. Detection of *E. coli* in various organs of infected birds using immunohistochemistry.

	Organs						
Group	Trachea	Air sac	Lung	Heart	Liver	Spleen	Bursa of Fabricius
Infected broiler	2/16[a]	3/16	9/16	3/16	4/16	6/16	10/11
Infected layer	1/16	0/16	3/16	1/16	0/16	3/16	8/12

[a]Number of positive samples / total number of samples tested.

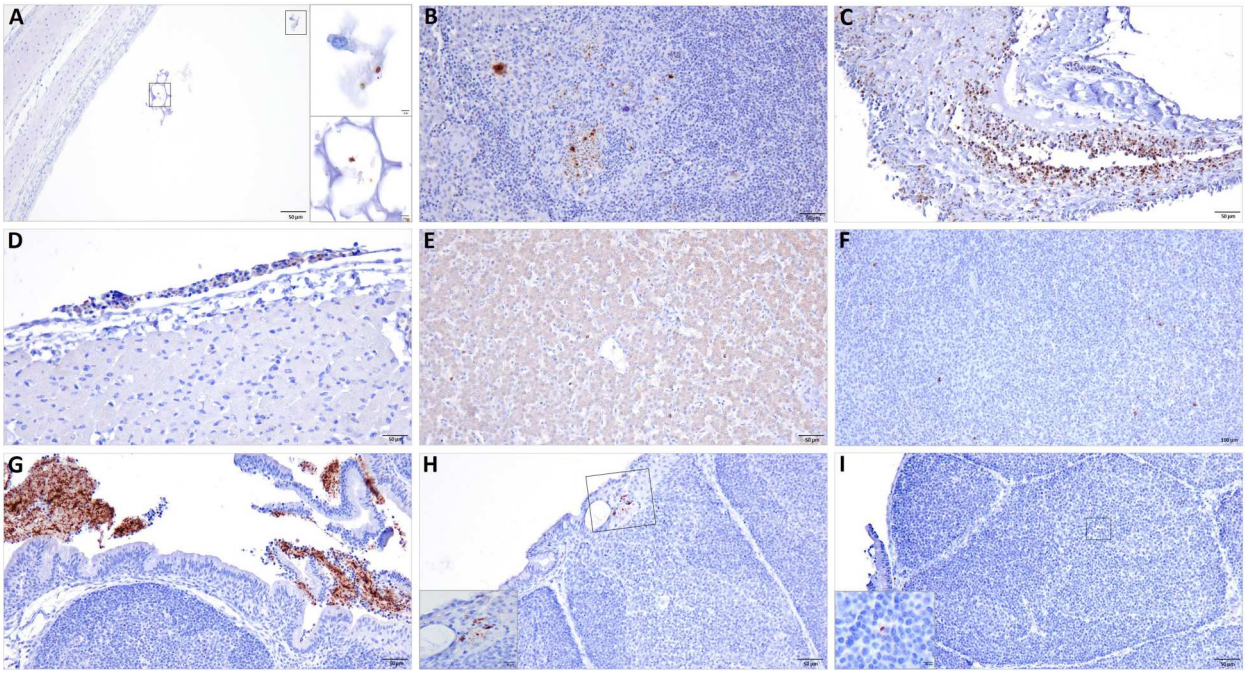

Figure 8. Immunostaining of *E. coli* in organs of APEC-infected birds. (A) Trachea, *E. coli* attached to the desquamated epithelium and in the secreted mucous (insets); (B) Lung, *E. coli* present in the area of inflammation; (C) Air sac, *E. coli* widely distributed throughout the epithelium; (D) Heart, individual *E. coli* bacteria present in the epicardium; (E) Liver and (F) spleen, *E. coli* antigens within their tissue parenchyma; (G, H, I) Bursa of Fabricius, DAB signal representing *E. coli* antigens colonized to the desquamated bursal epithelium and within the mucous exudate in the bursal lumen (G), in the follicle-associated epithelium (inset, H), and as an individual *E. coli* bacteria in the medulla of bursal follicle (inset, I).

lower clinical scores and macroscopic lesions in respiratory and systemic organs compared to birds in the infected broiler group. Less severe clinical presentation and macroscopic lesions in APEC-infected layer birds aligns with the result of an earlier study conducted by our group (Paudel *et al.*, 2021). In that study, 2-week-old SPF layer chicks aerosolized with the same APEC strain at a similar concentration showed no clinical signs with mild macroscopic lesions. In the current study, two birds from the infected broiler group were euthanized at 4 dpi due to neurological signs, yet no macroscopic or histological lesions were found in the brain. These signs could be attributed to the systemic inflammatory events induced by colibacillosis in other organs, as well as the production of bacterial endotoxins that alter the activity of purinergic and cholinergic enzymes involved in neurotransmission and cerebral immunomodulation (da Rosa *et al.*, 2020).

The increase in the spleen-to-bodyweight ratio during APEC infection has been well-documented in multiple studies (Bagheri *et al.*, 2023; Paudel *et al.*, 2023; Abdelhamid *et al.*, 2024; Shen *et al.*, 2024). In the current study, it was observed that the increase was significantly higher in broilers than in layers, which coincided with the severe macroscopic lesions, histologic lesions, and inflammation in the spleen of the infected broiler versus the infected layer. This outcome could be attributed to a higher systemic invasion rate of APEC in broilers, potentially reflective of differences in immune responses, as evidenced by the increased heterophil infiltration seen in the spleens of broilers.

Overall, the APEC infection resulted in more severe avian colibacillosis lesions and a higher re-isolation rate in broilers as compared to layers. Moreover, only the infected broilers tested positive for bacterial re-isolation from systemic organs. This increased susceptibility in broilers might be attributed to the prioritization of growth over immune defence mechanisms during selective breeding for higher bodyweight (Boa-Amponsem *et al.*, 1991; Praharaj *et al.*, 1997). Supporting this, another study has shown an inverse relationship between bodyweight and immune parameter levels such as antibody response and heterophilic/lymphocyte ratio in commercial broiler hybrids in response

to APEC infection via the air sac inoculation route, indicating that faster growth is associated with reduced immunity (Praharaj *et al*., 2002). These findings, combined with our findings, reinforce the hypothesis that genetic selection for growth and feed efficiency in broilers has inadvertently increased their vulnerability to colibacillosis (Gross, 1995; Yunis *et al*., 2000).

The current infection model used the genetically modified *ilux2-E. coli* PA14/17480/5/ovary strain. The insertion of the *ilux2* locus does not affect on the pathogenicity of the parent strain, and the tagged strain has been shown to be traceable in various organs of infected birds with clear discrimination from native commensal isolates (Abdelhamid *et al*., 2024). Employing the bioluminescence imaging of the whole bird carcass before the necropsy enabled us to uncover the unexpected bacterial colonization in the bursa of Fabricius. The bursa of Fabricius is a specialized organ in birds that plays a crucial role in the development and maturation of B lymphocytes, as well as in antibody production. However, it is not commonly considered as a sample material for bacterial re-isolation in routine diagnostics or in experimental studies examining the progression of avian colibacillosis in chickens, particularly those focusing on infections acquired via the respiratory route. Following the initial detection of *ilux*2-APEC in the bursa of Fabricius from a bird euthanized at 4 dpi, this organ was subsequently included in the analysis of all assessed parameters at later time-points. The detection of bioluminescence and the re-isolation of *ilux*2-APEC from the bursa of Fabricius in 58% of infected layers and 73% of infected broilers, along with its persistence up to 14 dpi, indicate that APEC exhibits a natural tropism for colonizing this organ. Interestingly, some birds tested positive for *ilux*2-APEC only in the bursa. This raises intriguing questions about how APEC reached the bursa, whether through the bloodstream or the bursal duct. While none of the intestinal samples exhibited bioluminescence and were unfortunately not assessed for the re-isolation of *ilux*2-APEC, the detection of *ilux*2-APEC on the walls of the box used for aerosol exposure indicates that aerosol droplets may have also settled on the chickens´ bodies, including their cloacal lips. This implies a possibility of cloacal uptake, as the cloacal lips of chickens exhibit a typical sucking reaction when touched or when in contact with a drop of liquid, known as cloacal drinking (Hu *et al*., 2004; Van der Sluis *et al*., 2009). Such behaviour could provide a potential pathway for the bacteria to enter through the bursal duct and into the bursal lumen (Ekino *et al*., 1985). Furthermore, alongside the re-isolation of APEC from infected birds for up to 14 dpi, the immunohistochemical analysis demonstrated the presence of APEC in the bursal lumen and/or the follicle-associated epithelium in almost all positive bursal samples. This suggest that APEC antigens were likely derived from the aerosol contaminated environment, got trapped within the bursal lumen, and were then taken up by the follicle-associated epithelium for transport into the bursal follicle (Ekino *et al*., 1995). Although APEC could persistently colonize the bursa of Fabricius, its colonization was asymptomatic and did not lead to any pathological lesions or inflammatory reaction, resembling a commensal relationship (Hermans *et al*., 2012; Dalmasso *et al*., 2023). However, the underlaying mechanisms allowing APEC to persistently colonize the bursa of Fabricius deserves attention in future studies. The unintentional detection of *K. pneumoniae* and *E. cloacae* in the bursa of Fabricius may facilitate APEC colonization through synergistic microbial interactions. Both *K. pneumoniae* and *E. cloacae*, members of the *Enterobacteriaceae* family alongside *E. coli*, share similar environmental preferences, thriving in neutral to slightly alkaline pH, and are considered a part of the normal avian gut microbiota (Christl *et al*., 1997; Ribeiro *et al*., 2023). Additionally, ability of *K. pneumoniae* to form mixed biofilms with *Proteus mirabilis*, often facilitated by *E. cloacae* and *E. coli*, suggesting that their presence in the bursa may similarly promote *E. coli* colonization and persistence through biofilm-associated mechanisms (Macleod & Stickler, 2007; Vuotto *et al*., 2014). Further research is needed to understand the evolution of the bursal microbiome and its relationship with gut microbiota dynamics.

In conclusion, broiler-type birds are more susceptible to developing avian colibacillosis, suggesting that their genetic background significantly impacts the susceptibility to APEC infections. This emphasizes the importance of effective management and targeted infection control measures in broiler farms to reduce the risk of exposure to APEC infections. The persistent colonization of APEC in the bursa of Fabricius, while all other organs remained negative, underscores the need for further investigation into the mechanisms of this localized infection following the aerosol exposure. Lastly, the aerosol infection model in broiler birds is a closer resemblance to spontaneous avian colibacillosis in field conditions, making it a preferred choice for further research, especially concerning preventive measures and disease mechanisms.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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