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Biochar litter amendment concerning environmental health and immunity in broilers

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Abstract

Background Environmental hygiene is an important factor in poultry production to keep broiler chickens' health and welfare. This study aimed to evaluate biochar-amended poultry litter to enhance environmental hygiene and broiler chickens' health and immunity by reducing bacterial load and improving environmental quality.

Method For 5 consecutive weeks, a total of 60 one-day-old Cobb broiler chickens were divided into three groups: a control group (CN) reared on sawdust litter, and two treated groups where sawdust litter was amended with 10% biochar (10%BC) and 20% biochar (20%BC) groups. BC was produced from poultry litter of the CN group through pyrolysis at 450 °C.

Results Total bacterial count, as well as *Escherichia coli* and *Salmonella* count in the air and litter environments, were weekly increased in the CN group. Biochar treatment, especially with the 10%BC group, significantly reduced bacterial counts. Hematological analysis revealed an elevated white blood cell (WBCs) count and a reduced heterophils/lymphocytes (H/L) ratio in the biochar-treated groups, indicating enhanced immune status and reduced stress compared to the CN group. Flow cytometry indicated significant increases in peripheral blood CD4 and CD8 T-lymphocytes, particularly with the 10%BC group. Serum immunoglobulins (IgA, IgY, IgM) were also released significantly with BC groups, especially 10% BC, compared to the CN group. Histopathological alterations in the spleen and thymus gland of broiler chicken biochar groups revealed structural improvements compared with the CN group.

Conclusion These findings suggested that biochar addition to poultry litter optimally enhances environmental hygiene, immune function, and overall broiler health, especially with 10%BC concentration. Thus, biochar represents a promising litter management strategy to mitigate bacterial load and improve poultry welfare.

Keywords Biochar, Broilers, Environmental quality, Flow cytometry, Immunoglobulins, Microbial load

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Introduction

The poultry industry is a key supplier of animal protein and white meat in Egypt and worldwide (Soliman and Hassan 2020). Poultry production can be affected by acute and chronic stressors throughout birds' lives, including nutritional, microbiological, management, and environmental factors (Campderrich et al. 2019; Ncho et al. 2025). Broiler chickens often spend their entire lives in contact with litter (Abougabal and Taboosha 2023). Therefore, litter quality is a major concern in chicken production because it can serve as a reservoir and vector for the transmission of infections (Gomes et al. 2022). Pathogenic or conditionally pathogenic microbes can cause allergic reactions, immunotoxins, and even infectious diseases (Chmielowiec- Korzeniowska et al. 2021).

Accumulation of bacteria in the air, on equipment, and in litter on poultry farms is one of the main factors that adversely affect industrial poultry performance (Valeris-Chacin et al. 2021). In addition, microbial breakdown of organic waste in litter and manure produces several gases, including ammonia, carbon dioxide, and volatile organic compounds (Jiang et al. 2023). *Escherichia coli* and *Salmonella sp.* are gram-negative bacteria belonging to the Family Enterobacteriaceae (Kumar et al. 2022). These species persist between production cycles, even after disinfection, and can cause health problems (Galgano et al. 2023), reduce productivity, and cause mortality because they can survive for extended periods in the poultry environment (Wibisono et al. 2020).

One of the main factors preventing broilers from reaching ideal development and feed rates is immunological stress, which seriously harms the chicken breeding sector (Bilal 2025). Several studies attributed this to a variety of variables, including living conditions and external stressors (Kumar et al. 2023), habitat alterations (Messina et al. 2018), hygienic quality of feed, water, and environment, as well as pathogen exposure (Wlaźlak et al. 2023). Chickens have CD4 and CD8 T-cell subsets, which are components of cellular immunity and are considered the most sensitive biomarkers among blood parameters. In addition, immunoglobulins (IgM, IgY, and IgA) are the three main classical immunoglobulins that are secreted by B-cells in birds (Mallaby 2022). Moreover, the spleen is the largest peripheral immune organ in the body, which is responsible for humoral and cellular immune functions (Chen et al. 2022). It can activate the immune response following interaction with pathogens (Null et al. 2024). In addition, the thymus gland is a central immune organ for the differentiation, selection, and proliferation of T-lymphocytes to functional T-lymphocytes (Xing et al. 2016; Ceccopieri and Madej 2024).

Different strategies can be used to maintain a healthy litter environment, reduce the risk of bacterial infections, and enhance overall bird performance, including regular litter amendments, adequate ventilation, and litter moisture control (Mocz et al. 2022; Ali et al. 2023). Biochar can improve animal performance, enhance gut health, and reduce ammonia emissions in livestock environments. Moreover, biochar has been investigated as an environmental amendment in livestock production due to its highly porous structure and adsorption capacity, which may influence the microbial activity and nutrient utilization. In addition, it has been reported to reduce greenhouse gas emissions and eliminate both organic and inorganic pollutants (Zaid et al. 2023; Weber et al. 2023; Kazemi 2025). The physicochemical properties of biochar play a crucial role in determining its adsorption capacity and environmental functionality. These properties typically include pH, specific surface area, pore structure, ash content, elemental composition (C, H, O, N), surface functional groups, and cation exchange capacity (CEC). Biochar generally exhibits an alkaline pH due to the presence of basic mineral components, which contribute to its ability to retain nutrients and improve environmental conditions (Ravindiran et al. 2024).

Therefore, this study aimed to evaluate some environmental hygiene factors in a poultry house and their effects on broiler chicken immune response, either through biochemical or histopathological investigations. In addition, the study was interested in evaluating the role of biochar added to poultry litter in different concentrations on the environment and birds.

Materials and methods

Experimental protocol of broiler chickens

The experimental broilers were reared under controlled microclimatic conditions throughout the study period. Ambient temperature was maintained at 32–34 °C during the first week and gradually reduced by 2–3 °C weekly until reaching 22–24 °C, while relative humidity was kept between 55 and 65%. Birds were fed a commercial corn–soybean–based diet formulated according to NRC recommendations, with starter and grower rations meeting the required nutrient specifications. Feed and water were provided ad libitum through an automatic feeder and nipple drinker system. Drinking water was sourced from the municipal supply and regularly monitored for physicochemical quality parameters. A lighting program of 23 h light and 1 h dark was applied during the first week, followed by 20 h light and 4 h dark thereafter, using white LED lighting at appropriate intensity. Ventilation was provided through a natural ventilation system through a window (50 cm x 45 cm), ensuring adequate air exchange and ammonia control. Strict biosecurity measures were implemented, including insect- and rodent-proofing of the facility. A routine cleaning and disinfection protocol was followed before chick placement. No vaccination and no therapeutic treatments were administered to birds.

All the experimental studies have been approved by the Mansoura University, Egypt, MU-ACUC Animal Care Committee (SC. PhD.23.02.4).

Control broiler group

A number of 20 Cobb breed broiler chickens of one day old were used as control (CN) group, housed in an experimental room of 3.5 m length x1.5 m width x 3 m height. Sawdust was utilized as the bedding material, which was added daily through the experimental period of 5 weeks. The untreated poultry litter of the CN group was collected at the end of the experimental time and utilized for producing biochar for the next two experimental broiler chicken groups.

Poultry litter and biochar production

Poultry litter (PL) of the CN group was collected while avoiding feathers as much as possible. The collected PL was dried in the sun for three days to get rid of excess moisture and stored in plastic pots, then transferred to the laboratory until use. PL was subjected to pyrolysis in a muffle furnace under limited oxygen for biochar (BC) production, where crucibles were filled with PL residue, then covered. A JUNG muffle furnace at 450 °C was used for half an hour for the BC manufacturing processes, according to the method described by Pereira et al. (2019).

Biochar broiler groups

For the same number, age, experimental period, and environmental conditions as in the CN group, another two Cobb broiler chicken groups were raised for 5 weeks. Regular addition of BC, incorporated into the litter, was performed as 10% of the total amount of the bedding sawdust (10%BC group). However, in the other group, 20% of the bedding material was added as BC (20%BC group).

Environmental hygiene investigations

CO_2 levels and C%

The level of air carbon dioxide (CO_2) was assessed using a digital Perfect Prime CO_2 390 CO_2 Home Use Meter. Moreover, litter carbon (C)% was detected according to the method of AOAC (2019), where 2 g of air-dried PL were placed in a silica crucible and ignited at 600 °C in a muffle furnace till a constant weight, then the percentage of carbon content was calculated.

Bacterial examination of air and litter

Air analysis for bacterial load was conducted using the sedimentation method described by Kalwasińska et al. (2012). For each group, 3 open Petri dishes with a respective medium plate count agar for total bacterial count (TBC), Eosin methylene blue for *Escherichia coli* (*E. coli*), and XLD (Xylose Lysine Deoxycholate) agar for *Salmonella* were exposed weekly to air samples for 30 min, then closed and incubated at 37 °C for 24 h. All colonies grown on plate count agar were counted; meanwhile, greenish metallic colonies only were counted on EMB, and red colonies with black centers or completely black colonies were counted on XLD and expressed as (CFU/Plate/min).

However, for litter examination, TBC was detected utilizing the pour plate technique according to Mohammed and Elbably (2020). About 5 g of each collected litter sample was suspended in 30 mL of tryptone soya broth (TSB) (Oxoid, Hampshire, United Kingdom) and mixed well for 5 min. Then, tenfold serial dilutions were conducted. 0.1 µL of each 6–9 dilution was added to two sterile petri dishes with plate count agar (Oxoid, Hampshire, United Kingdom) to determine the TBC of each sample. The plates were incubated at 37 °C for 24 h, and the total number of colonies on each plate was counted (CFU/g), as stated by Khaskheli et al. (2025).

Moreover, *E. coli* and *Salmonella* were isolated using the methods described by Roll et al. (2011). For *E. coli* isolation, about 25 g of each litter sample was weighed and homogenized with 225 ml of buffered peptone water (Oxoid, Hampshire, UK). These mixtures were incubated at 37 °C for 24 h. A loopful of every diluted sample was cultured on eosin methylene blue agar media (EMB agar, Modified Levine Thermo ScientificTM OxoidTM CM0069B, weight 500 g) and incubated at 37 °C for 24 h (Mohammed and Elbably 2020). Presumptive colonies of *E. coli* had a yellow-green metallic sheen on eosin methylene blue agar (Islam et al. 2014). However, for *Salmonella* isolation, about 1 ml of each diluted sample was mixed with 10 ml of Rapaport Vassiliadis (RV) broth (Oxoid, Hampshire, United Kingdom) and incubated at 42 °C for 24 h for enrichment. Then, a loopful of each broth was cultured on Xylose Lysine Deoxycholate (XLD, Oxoid, Hampshire, United Kingdom) plates and incubated for 24 h at 37 °C. Presumptive *Salmonella* colonies were pink with a black center colony on XLD media. After that, *E. coli* and *Salmonella* were detected biochemically by using the following tests: indole, Methyl Red, Voges-Proskauer, citrate utilization, and triple sugar iron test (Oxoid, Hampshire, United Kingdom) (Tilahun and Efa 2026).

Broiler chicken investigations

Collection of blood and tissue samples

At the end of the experimental period, 30 blood samples were collected (10 samples for each group) and each sample was divided into 2 parts. The first one was collected on EDTA to determine total and differential leucocytes (WBCs) count, as well as T-lymphocytes surface markers (CD4 and CD8). The second sample was taken on a sterile tube without an anticoagulant and centrifuged using

a benchtop centrifuge (Centurion Scientific Ltd., United Kingdom) at 1500 rpm for 15 min to separate serum for determining immunoglobulins (IgA, IgY, and IgM). After that, broiler chickens were dissected to obtain the spleen and thymus gland for histological examination.

Estimation of total and differential count of leucocytes

Blood samples were analyzed using the automatic hematological analyzer (Dymind DH36, China) for assessment of total and differential WBCs count that includes heterophil (H%), lymphocytes (L%), monocytes (M%), basophils (B%), and eosinophils (E%). The H/L ratio was calculated by dividing the number of heterophils by the number of lymphocytes.

Estimation of T-lymphocyte surface markers

EDTA blood was used for isolating peripheral blood mononuclear cells (PBMCs). Blood samples collected from each group were diluted 1:1 with phosphate-buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation according to the method of Dalgaard et al. (2010), using Ficoll–Hypaque density 1.077 g/ml (Sigma Aldrich). Briefly, 10 mL of diluted blood was carefully layered on top of Ficoll–Hypaque in a tube and then centrifuged for 20 min at 400 g, causing mononuclear fractioning by Ficoll–Hypaque density gradient separation. The cells in the interphase layer were aspirated and washed three times. Then, the supernatant was removed, and a pellet of PBMCs was collected. The cells were resuspended and then adjusted to a concentration of 1×10^6 cells/ml with PBS for further assays.

Flow cytometric analysis of PBMCs was briefly achieved on 50 µL (1×10^6 cells) that was incubated for 30 min at 4 °C in a dark place with 10 µL mouse anti-chicken CD4- FITC (Southern Biotech, Catalog number: 8210-02) or 10 µL mouse anti-chicken CD8-FITC (Southern Biotech, Catalog number: 8220-02). The percentage populations of CD4 and CD8 lymphocytes in the PBMCs were analyzed using a flow activated cell sorter (FACS) caliber flow cytometer (Becton Dickinson, Sunnyvale, CA, USA), as described by Dean and Jett (1974). Viable cells were gated on morphological criteria (FSC/SSC) in order to eliminate cellular debris and damaged cells. The CD4 and CD8 positive cells were then detected on the viable population.

Estimation of immunoglobulins

Serum immunoglobulins were determined using chicken IgA, IgY, and IgM Elisa kit (MyBioSource, San Diego, USA), Catalog numbers: MBS564152, MBS260043, and MBS706158, respectively.

Histological studies

Hematoxylin and eosin (H&E) staining was used to evaluate structural alterations in the spleen and thymus gland under a light microscope (Nikon ECLIPSE Si). According to Stinnett's (2015) method, the tissues were immediately fixed in 10% formalin after dissection for 72 h, then were embedded in paraffin wax for sectioning at 5 µm thickness and staining.

Statistical analysis

The GraphPad Prism software version 5.04, Inc., La Jolla, CA, USA) was used for data analysis. One-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons was applied to all parameters. Data were expressed as mean± standard deviation (SD), where significance was considered at $p < 0.05$.

However, the interaction between air and litter environmental hygiene measurements, as well as the biochemical parameters were clarified by using Pearson correlation coefficient (r).

Results

Environmental hygiene investigations

CO_2 levels and C%

Through the period of investigation, CO_2 levels in the air environment and C% in the litter showed an irregular pattern, where BC groups recorded elevations of both compared to the untreated CN group. The lowest CO_2 levels were detected in the 1st week of the experiment for CN, 10%BC, and 20%BC groups. However, the highest CO_2 levels were recorded for the CN group in the 3rd week, while the 10%BC and 20%BC groups in the 4th week. Results also showed a significant increase in CO_2 levels with the BC groups compared to the CN group. Similarly, the lowest C% was obtained in the 1st week, while the highest was observed in the 5th week for CN, 10%BC, and 20%BC groups. Although C% was increased in the BC groups, this increase was not significant compared to the CN group (Table 1).

Bacterial examination of the air and litter environments

Regarding bacterial contamination as a part of environmental hygiene parameters, the obtained data showed a gradual increase through the investigation period in bacterial load either in the air or litter environment of the three investigated groups. The lowest air TBC, *E. coli*, and *Salmonella* counts were observed in the 1st week for all groups, while the highest counts were detected in the 5th week. However, BC groups showed a mostly significant reduction in bacterial load through the study period compared to the untreated CN group.

Similarly, for all investigated groups, the lowest litter bacterial count was detected in the 1st week; however, by increasing the experimental time, and at the end of

Table 1 Weekly changes in the air and litter environmental hygiene parameters in the investigated groups

Weeks + Groups	1st week			2nd week			3rd week			4th week			5th week		
Parameters	**CN**	**10%BC**	**20%BC**	**CN**	**10%BC**	**20%BC**	**CN**	**10%BC**	**20%BC**	**CN**	**10%BC**	**20%BC**	**CN**	**10%BC**	**20%BC**
CO_2 (ppm) (air)	1489 ±61.33	1757^{a}±64.91	1791^{a}±65.70	2773 ± 69.00	2289^{a}± 30.28	2315^{a} ± 32.49	4985 ± 11.98	3777^{ab} ± 26.19	3546^{a} ± 35.69	2258 ± 27.24	4165^{a} ± 23.68	4170^{a} ± 26.42	1503 ±5.67	3085^{ab} ± 17.42	2614^{a} ± 26.72
T.B.C (air)	90.0 ±8.52	56.3^{ab}± 11.1	77.3 ± 10.7	110 ± 18.1	74.4^{a} ± 14.8	88.3^{a} ± 15.6	150 ± 2.00	82.0^{a} ± 14.3	92.3^{a} ± 11.2	164 ±6.00	97.5^{a} ± 21.0	112^{a} ± 12.7	179 ± 22.8	125^{a} ± 19.3	135^{a} ± 19.3
E. coli (air)	4.25 ± 1.71	2.00 ± 1.00	3.67 ± 2.08	6.00 ± 0.00	3.00 ± 2.65	5.00 ± 3.00	8.75 ± 5.38	6.75 ± 2.87	7.00 ± 2.58	37.7 ± 4.73	11.0^{ab} ± 2.65	27.0 ± 5.57	55.0 ± 7.00	18.0^{a} ±4.00	33.5^{a} ± 9.81
Salmonella (air)	2.00 ± 1.15	0.00^{a} ± 0.00	0.00^{a} ± 0.00	3.00 ± 0.00	0.00^{a} ± 0.00	0.00^{a} ± 0.00	18.0 ± 4.00	6.00^{a} ± 2.00	10.0 ± 3.61	20.0 ± 4.90	7.25^{a} ± 3.77	9.00^{a} ±4.08	31.0 ± 6.56	17.0 ± 5.00	21.8 ± 7.93
C (%) (litter)	5.28 ± 1.32	7.71 ± 1.59	7.83 ± 1.05	10.2 ± 1.72	11.5 ± 2.32	13.5 ± 2.33	11.3 ± 2.50	11.90 ± 2.02	13.1 ± 2.14	11.20 ± 3.86	12.5 ± 2.27	12.8 ± 2.29	11.71 ± 2.30	13.8 ± 2.69	14.6 ± 2.47
T.B.C ($x10^6$) (litter)	151 ± 8.54	145 ± 13.2	150 ± 29.1	170 ± 18.0	149 ± 27.6	157 ± 24.9	176 ± 10.1	152 ± 30.6	166 ± 14.4	182 ± 20.7	158 ± 10.7	172 ± 6.24	194 ± 2.65	162^{ab} ± 12.6	187 ± 7.94
E. coli ($x10^6$) (litter)	15.0 ± 4.36	5.33^{a} ± 1.53	8.00 ± 1.73	45.0 ± 11.1	28.0 ± 6.56	30.0 ± 8.89	49.0 ± 8.00	31.7 ± 6.43	34.3 ± 9.45	54.0 ± 13.9	36.7 ± 8.02	45.0 ± 13.2	75.0 ± 7.87	55.0^{a} ± 8.89	60.5 ± 6.86
Salmonella ($x10^6$) (litter)	9.33 ± 1.53	3.50^{a} ± 1.91	7.50 ± 2.38	16.7 ± 5.69	5.00^{a} ± 1.00	12.0 ± 4.36	26.7 ± 9.29	14.0 ± 3.61	19.0 ± 6.24	34.0 ± 7.94	18.0^{a} ± 5.20	26.0 ± 3.61	39.7 ± 8.02	21.0^{a} ± 8.54	32.5 ± 5.69

Values are represented as mean ± SD. CN: control, 10%BC: litter with 10%biochar, 20%BC: litter with 20% biochar. a: significance between BC groups and CN, b: significance between the two BC groups ($p<0.05$)

Table 2 Pearson-moment correlation (r) between bacterial load, C% in litter and CO2 in air samples of investigated groups

Litter	*E. coli*	*Salmonella*	TBC	C%
Air				
E. coli	0.815***	0.887***	0.770***	0.244
Salmonella	0.880***	0.883***	0.774***	0.311
TBC	0.849***	0.942***	0.805***	0.298
CO_2	0.141	0.177	-0.022	0.543*

* = Significant at $p \leq 0.05$ ** = Significant at $p \leq 0.01$ *** = Significant at $p \leq 0.001$

Table 3 Total and differential count of WBCs in the blood of different investigated broiler chicken groups

Groups	CN	10%BC	20%BC
Parameters			
WBCs (*10/mm^3)	88.60 ± 8.30	107.30^{a} ± 8.23	101.90^{a} ± 6.68
H (%)	2.50 ± 0.58	1.25^{a} ± 0.50	1.60 ± 0.55
L (%)	90.00 ± 0.71	94.50^{a} ± 1.73	92.60 ± 2.07
H/L ratio	0.040 ± 0.01	0.015^{a} ± 0.01	0.017^{a} ± 0.01
M (%)	5.40 ± 0.55	2.60^{a} ± 0.89	3.75^{a} ± 0.96
E (%)	1.40 ± 0.55	1.20 ± 0.45	1.20 ± 0.45
B (%)	0.20 ± 0.45	0.40 ± 0.55	0.40 ± 0.55

Values are represented as mean ± SD. CN: control,10%BC: litter with 10%biochar, 20%BC: litter with 20% biochar. a: significance between BC groups and CN, b: significance between the two BC groups ($p<0.05$)

the experiment (5th week), the highest bacterial load was observed for TBC, *E. coli*, and *Salmonella*. In the 5th week, especially 10% BC group showed a significant decrease in TBC compared to the CN group or 20% BC group. However, the decrease in *E. coli* was significant compared to CN group in the 1st week as well as the 5th week. Furthermore, *Salmonella sp.* decreased significantly with 10%BC group compared to CN group in the 1st, 2nd, 4th, and 5th weeks (Table 1).

Pearson correlation coefficient (r) showed a very highly significant positive correlation between air and litter TBC, *E. coli*, and *Salmonella* counts. In line with these results, litter C% was positively significant correlated with CO_2 in the air (Table 2).

Total and differential WBCs count

Table 3 illustrates the total and differential WBC counts of the untreated CN broiler chicken group and the other treated BC groups. The obtained data showed the BC groups, especially the 10% BC group, with a significant elevation in total WBCs count along with L%, accompanied by a marked decrease in H%, H/L ratio, and M% compared to the CN group. Moreover, the change in E% and B% was non-significant in both BC groups compared to the CN group. It should also be mentioned that no significant difference was noticed between the two investigated BC groups, either in the total or differential count of WBCs.

T-lymphocyte surface markers and Immunoglobulins

The cellular immune response was mediated by T-lymphocyte surface markers (CD4 and CD8). The data in Table 4 showed a reduction in the values of both markers in the peripheral blood of the CN group. However, the immune markers were significantly elevated in both BC groups compared to the untreated CN group. Furthermore, the increase in the 10%BC group was significant compared to the 20%BC group. Similarly, the levels of serum immunoglobulins (IgA, IgY, and IgM) mediated by B-lymphocytes in the CN group recorded low levels. However, the BC groups, especially 10%BC, showed a significant increase in immunoglobulins compared to the CN group. Moreover, the 10%BC group showed significant increases in IgM compared to the 20%BC group.

Correlation analysis using Pearson correlation coefficient, as seen in Table 5 showed a significant negative correlation between air CO_2 and blood E%, along with air and litter TBC and CD4%, as well as CD8%. In addition to a significant negative correlation between TBC in litter and serum IgM.

Table 4 T-lymphocytes surface markers and immunoglobulins of different investigated broiler chicken groups

Groups	CN	10%BC	20%BC
Parameters			
CD4 (%)	33.20 ± 3.52	59.63 ^{ab} ± 0.80	52.07 ^{a} ± 2.90
CD8 (%)	40.33 ± 1.75	68.90 ^{ab} ± 3.55	60.07 ^{a} ± 4.19
IgA (mg/dl)	11.50 ± 1.50	16.33 ^{a} ± 2.38	13.20 ± 0.99
IgY (mg/dl)	56.80 ± 6.62	74.83 ^{a} ± 7.81	64.00 ± 5.68
IgM (mg/dl)	6.41 ± 1.26	12.16 ^{ab} ± 1.73	8.36 ± 1.60

Values are represented as mean ± SD. CN: control,10%BC: litter with 10%biochar, 20%BC: litter with 20% biochar. a: significance between BC groups and CN, b: significance between the two BC groups ($p<0.05$)

Histological studies

Spleen

The present study showed the spleen of the untreated CN group (Fig. 1a, a1) with severe damage in the architecture, represented by dysplastic white pulp, which was indistinct from the red pulp. Congested blood vessel and sinusoidal capillaries were noticed along with severe depletion in lymphocytes. Additionally, hemorrhage and hemosiderosis were observed. Besides, apoptotic lymphocytes with nuclear debris in white pulps were also found in the sections of spleen from the untreated CN broiler chickens. On the other hand, the BC groups, especially in 10% BC group, showed an obvious improvement in spleen structure (Fig. 1b, b1) where distinct red and white pulp appeared in addition to the presence of a lymphatic nodule and a capsule surrounding the spleen. However, the 20%BC group appeared with mild depletion of lymphocytes along with distinct red pulp and white pulp (Fig. 1c, c1).

Thymus gland

Histological investigations of the broiler chicken's thymus gland in the untreated CN group are illustrated in Fig. 2a, a1. A degenerative connective tissue capsule surrounded less differentiated thymic lobules, where the medulla occupies a large area, was observed. In addition to the appearance of undistinguished inter-tubular connective tissue septa and the presence of moderate to severe atrophy with lymphoid depletion in cortical lymphocytes (massive lymphocytolysis). Atrophic changes were seen both in the cortex and medulla led to a thinning of the cortex that appeared like medulla. On the other hand, the BC groups showed significant improvement in the thymus gland structure. The 10%BC group (Fig. 2b, b1) showed a well-developed thin connective tissue capsule and thymic lobules with distinct cortex and medulla, noticed inter-tubular connective tissue septa, and less lymphocytolysis. Moreover, Hassall's corpuscles clearly appeared either in the cortex or medulla in 10%BC group. Whereas, the 20% BC group (Fig. 2c, c1) showed less degenerated connective tissue capsule, inter-tubular

Table 5 Pearson-moment correlation (r) between air, litter environmental hygiene parameters, and immune investigations

Environ	TBC		E. coli		Salmonella		CO_2	C%
Parameters	**Air**	**Litter**	**Air**	**Litter**	**Air**	**Litter**	**Air**	**Litter**
WBCs	−0.221	0.010	-0.167	0.003	-0.196	-0.157	−0.061	0.063
H%	0.130	0.063	0.058	-0.092	-0.049	0.046	−0.286	-0.377
L%	−0.240	−0.074	0.045	0.114	0.125	-0.091	0.037	0.184
M%	0.320	0.095	0.005	-0.037	-0.029	0.170	0.042	-0.095
B%	−0.098	−0.233	0.062	−0.335	−0.165	-0.014	−0.004	-0.307
E%	−0.044	0.019	0.032	0.005	-0.127	-0.081	−0.539*	−0.190
CD4	−0.557*	−0.634*	-0.354	-0.254	-0.348	-0.462	0.221	0.328
CD8	−0.553*	−0.621*	-0.305	-0.206	-0.351	-0.430	0.158	0.329
IgA	−0.182	−0.144	-0.055	0.115	0.081	-0.092	0.054	0.203
IgY	−0.049	0.071	0.059	0.134	0.278	-0.027	0.066	-0.054
IgM	−0.451	−0.564*	-0.156	-0.194	-0.206	-0.320	0.073	-0.032

* = Significant at $p \leq 0.05$ ** = Significant at $p \leq 0.01$ *** = Significant at $p \leq 0.001$

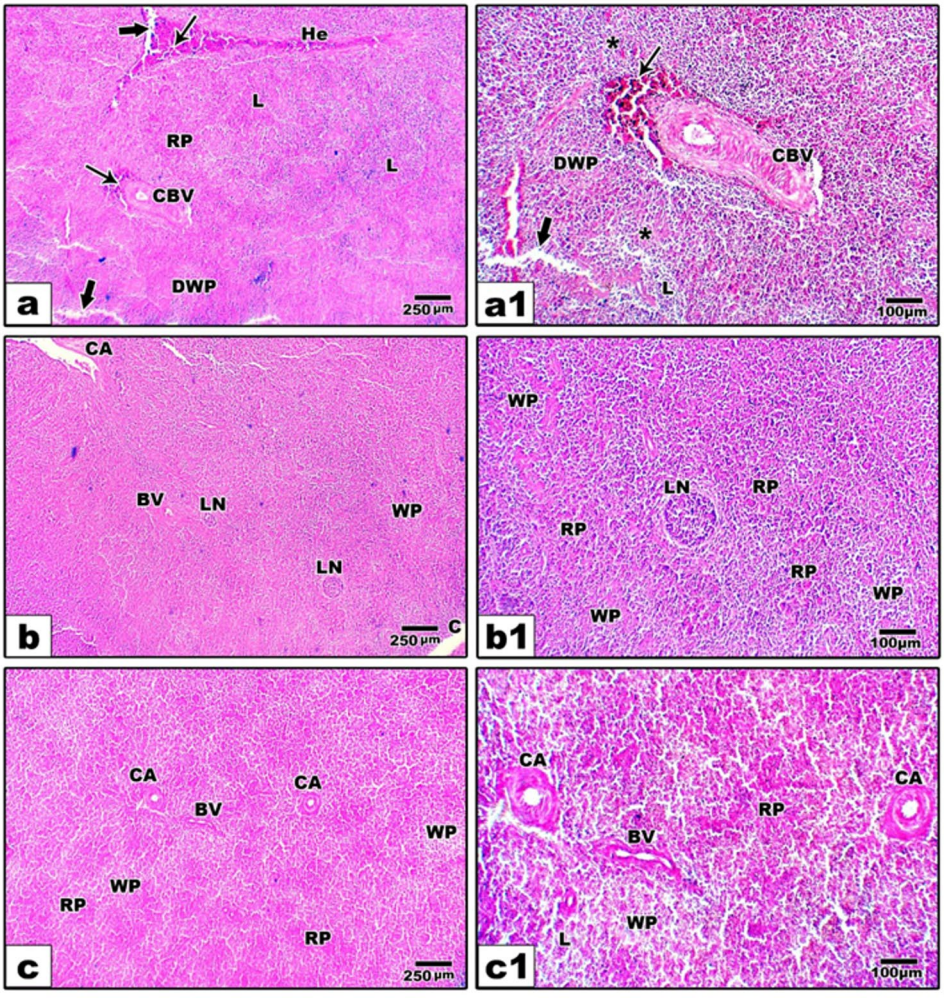

Fig. 1 Photomicrographs of histological sections of the spleen stained by H&E, showing control group (**a**, **a1**) with dysplastic white pulp (DWP) indistinct from the red pulp (RP), congested blood vessel (CBV), congested sinusoidal capillaries (star), small clumps and sever depletion of lymphocytes (L), hemorrhage (He), hemosiderosis (arrow) and apoptotic lymphocytes with nuclear debris in white pulps (thick arrow). 10%BC group (**b**, **b1**) showing normal white pulp (WP) distinct from the red pulp (RP), clearly distinguished, thick capsule covers the spleen (C), central artery (CA), lymphatic nodule (LN), blood vessel (BV). 20%BC group (**c**, **c1**) showing red pulp (RP) and white pulp (WP) with mild depletion of lymphocytes (L), central artery (CA), and blood vessel (BV) appeared

connective tissue septa, and thymic lobules with the presence of little lymphocytolysis.

Discussion

Poultry litter is characterized by potential biochar production (Pereira et al. 2019). Biochar was produced in the present study through pyrolysis at 450 °C, where, above this temperature, biomass degradation did not exceed 10% (Pereira et al. 2021). Moreover, several studies referred to biochar as a carbon-rich material (Pontiroli et al. 2019; Tsai and Chang 2021; Wani et al. 2025). This may explain the non-significant difference between CN and BC groups in litter C%, as well as CO_2 in the air. Chen et al. (2020) and Chung et al. (2021) agreed with this observation, recording generally increased emissions of CO_2 from manure when biochar was used for co-composting with manure. In addition, Czekala et al. (2016) reported that biochar consists of 40% carbon, so increased BC% in litter could elevate C-CO_2 levels in poultry litter. This may explain increased air CO_2 levels and litter C% in the 20%BC group compared to the 10%BC group.

The present study also investigated bacterial load in the air and litter, as an additional environmental hygiene parameter. The untreated CN group reared on ordinary poultry litter showed elevated TBC, as well as *E. coli* and *Salmonella* count in the air and litter environment. Diaz et al. (2019) and Plumblee Lawrence et al. (2022) attributed the accumulation of bacteria in poultry litter to the poultry gut, which is full of a variety of microbes released with broiler droppings to contaminate and accumulate in the litter. In turn, Gržinić et al. (2023) and Hemavarshini et al. (2025) linked the dominance of bacteria to the fecal

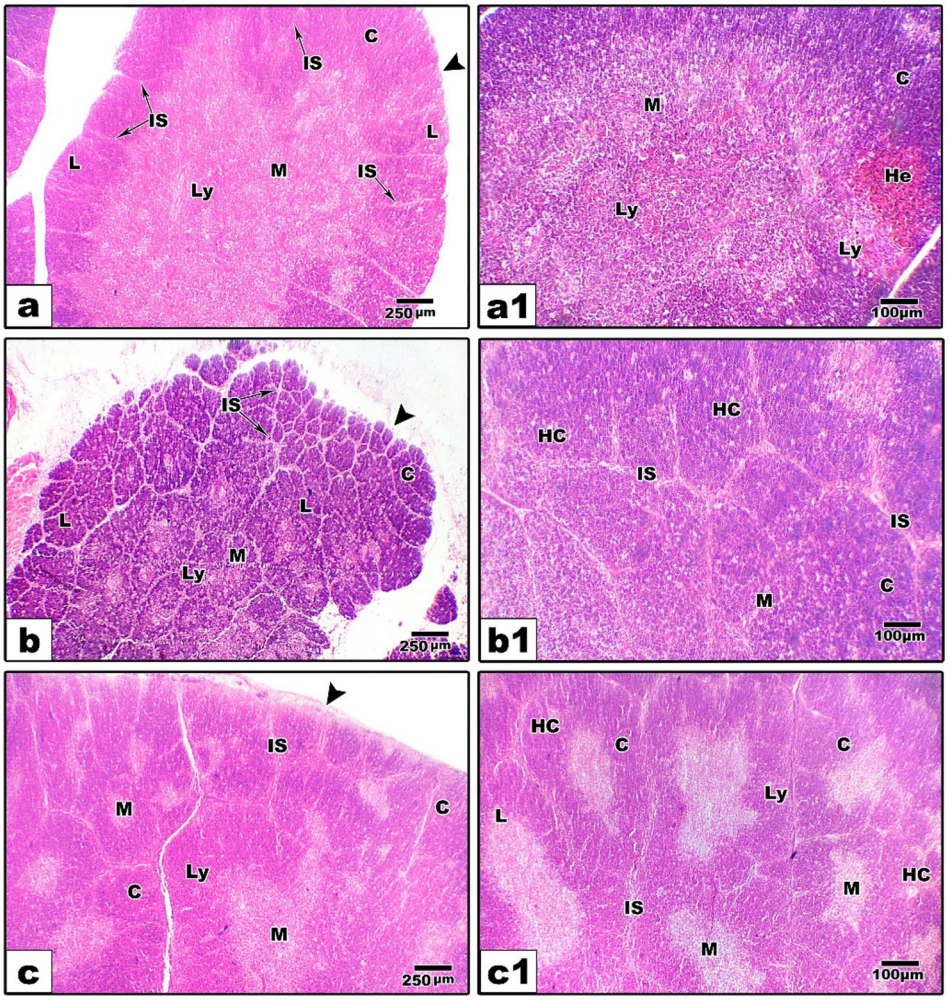

Fig. 2 Photomicrographs of histological sections of the thymus gland of untreated CN group (**a**, **a1**) showing degenerated connective tissue capsule (arrowhead) and disrupted and undistinguished thymic lobules (L), cortex (C), medulla (M), and inter-tubular connective tissue septa (IS). Massive lymphocytolysis (Ly), eosinophilic debris with hemorrhage (He). 10%BC group (**b**, **b1**) showing well-developed thin connective tissue capsule (arrowhead) and thymic lobules (L), cortex (C), medulla (M), inter-tubular connective tissue septa (IS), Hassall's capsule (HC). 20%BC group (**c**, **c1**) showing less degenerated connective tissue capsule (arrowhead) and thymic lobules (L), cortex (C), medulla (M), inter-tubular connective tissue septa (IS), Hassall's Capule (HC), hemorrhages (He), lymphocytolysis (Ly). Hematoxylin and Eosin (40X-100X)

matter dispersed by the birds. On the other hand, Douglas et al. (2025) attributed the presence of these organisms to contamination from the outside environment, possibly during the bird feeding.

The present study revealed that the addition of BC to PL revealed a pronounced reduction in the bacterial growth either in the air or litter environments. These findings agreed with the studies of Mohammadi-Aragh et al. (2021 and 2023), who attributed this to the increased pyrolysis temperature during BC production, which results in more stable aromatic structures that are difficult for microorganisms to catabolize. Additionally, the reducing effect of BC on bacterial growth was mostly pronounced in the 10%BC group compared to the 20%BC group. Linhoss et al. (2023) attributed this to the litter moisture content, which is an important factor for microbial and pathogen growth in broiler litter. Our previous study, Zaid et al. (2023), recorded a significant decrease in litter moisture with the 10%BC group compared to the 20%BC group and CN group, attributing this to the higher holding capacity of 10%BC than 20%BC. Kamyab et al. (2025) agreed with this notification, suggesting that increasing BC concentration does not necessarily enhance its antibacterial efficacy.

The present study was also extended to show pronounced alterations in broiler chicken health, especially the immune status related to bacterial load in the environment. Leukocytes are one of the hematological parameters that have an important role in the immune response and are implicated in several inflammatory processes (Staley et al. 2018; Nwaigwe et al. 2020). The current data showed a significant decrease in the total WBCs

count in blood samples from the untreated CN group compared to the BC groups. Wani et al. (2015) referred to this decrease as an indication of birds' immune suppression due to bacterial infection. Moreover, the recorded increase in H/L ratio in the untreated CN group provides valuable information on the reduced immune status of birds, as well as their acute and chronic conditions in terms of stress physiology and welfare (Sokolenko et al. 2024). Adeke et al. (2023) supported this through associating the increase in H/L ratio with environmental hygiene variables, including bacterial load contamination, which could affect the immune status of broiler chickens.

In this regard, broiler chickens have CD4 and CD8 T cell subsets, which are components of cellular immunity. CD8 T cells play a vital role during cytotoxic responses by killing infected target cells, whereas CD4 T cells help in eliminating pathogens (Alvarez et al. 2020). The current study showed a noticeable decrease in both CD4 and CD8 levels in the blood of the untreated CN group compared to the treated BC groups. This was in accordance with the study of More-Bayona et al. (2020), who explained that pathogenic microorganisms can cause an increase in macrophages, decrease the number of CD8 lymphocytes, and promote inflammatory reactions in the body. Roque et al. (2015) explained this as broiler chickens exposed to higher endotoxin levels have a lower percentage of lymphocytes, indicating a negative association between endotoxin levels and cell-mediated immunity in broiler chickens. At the same time, the current data showed a significant decrease in serum immunoglobulins (IgA, IgY, and IgM) in the CN group compared to the BC groups. This may be due to the increased bacterial growth in the CN group, which induces many endotoxins that are found in the outer membrane of gram-negative bacteria such as *E. coli* and *Salmonella* and cause immunologic modulations (Shaji et al. 2023).

Such disturbance in the immune status of the untreated CN group could be attributed to the damage of the immune organs, including the spleen and thymus gland. The present study supported these foundations through histopathological investigations in the spleen and thymus gland of different groups. The observed damage of the splenic and thymus gland tissues in the untreated CN group was compatible with the biochemical deviations in the measured immunological parameters. Dar et al. (2019) attributed this to severe *Salmonella* infection that might result in hemorrhagic splenic lesions. Additionally, Shah et al. (2020) reported that the thymus is extremely susceptible to stress, and its function can be impacted by a variety of environmental contaminants.

The pronounced decrease of bacterial load in BC groups compared to the CN group was accompanied by a significant increase in WBCs count, and a marked decrease in H/L ratio, indicating an effective role of BC addition, especially 10%BC, in decreasing stress responses in the birds (Birhan 2019; Zhang et al. 2023). Moreover, BC groups showed marked elevation in T-lymphocytes (CD4 and CD8) and immunoglobulins. This may be due to the decrease in bacterial load, which is captured by carbon in biochar as a carbon-rich renewable biomaterial used in various environmental remediation applications, such as eliminating both organic and inorganic pollutants (Gwenzi et al. 2021). This was agreed with Enyenihi et al. (2022), who reported that carbon can adsorb microorganisms such as *Salmonella* and *E. coli*.

In turn, the current study recorded a pronounced alleviation in the histological structure of both immune organs (spleen and thymus) with BC groups, especially 10%BC group, compared to the CN group. This was in accordance with Rashidi et al. (2020), who revealed that the role of BC in decreasing bacterial load participates in enhancing immune organs and immune response.

Conclusion

This study confirmed that poor environmental hygiene, particularly high bacterial contamination in air and litter, contributes to immunosuppression in broiler chickens, as reflected by alterations in leukocyte counts, CD4, CD8, immunoglobulins, and histopathological changes in immune organs. The addition of BC, especially at 10%, effectively reduced these adverse effects and improved immune status and overall health. These findings emphasize the importance of litter management and support the use of BC as a practical strategy to enhance broiler performance and immunity.

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Author contributions

H.A.Z. collected the data and analyzed them. H.T.E participated in reviewing the manuscript and analyzing data. M.A.E reviewed the manuscript. M.M.E suggested the study point and reviewed the manuscript. A.E.A suggested the study design, reviewed the manuscript, and interpreted data. All authors contributed to the study conception and design.

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Data availability

All data used during this study are available in the submitted article.

Declarations

Ethics approval and consent to participate

All the experiment studies have been approved by the Mansoura University, Egypt, MU-ACUC Animal Care Committee (SC. PhD.23.02.4).

Consent for publication

All the listed authors approved the final version of this manuscript and consent to its submission for publication. We affirm that the manuscript is original, has not been published elsewhere, and is not under consideration by any other publication. We also declare that all data, images, or other materials included in the manuscript, where applicable, have been obtained with proper authorization and that necessary permissions have been secured for their publication. By granting this consent, we accept the journal's policies regarding copyright, licensing, and publication ethics.

Competing interests

The authors declare no competing interests.

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