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Sustainable Broilers Production Performance under High Stocking Condition through Colocynth Seed Supplementation

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Abstract: The negative impacts of high stocking density on the commercial poultry industry compromise sustainable birds' productivity and welfare. Thus, we investigated the potential of colocynth seed (CC) supplementation in alleviating the negative impacts of high stocking density on broilers' performance, immunity, inflammation, and redox status. A total of 648 one-day-old male Cobb 500 chicks were recruited and distributed into 2 × 2 factorial arrangements. The treatment groups were assigned based on stocking density as low stocking density (9 birds/m^2; LSD) or high stocking density (19 birds/m^2; HSD), and CC supplementation as without (0 g/kg feed; −CC) or with (1 g/kg feed; +CC) supplementation. Data were collected from week three to week five of age. Production performance was monitored and meat quality was assessed. Blood samples were collected to measure stress markers, humoral immune response, inflammatory cytokines, and antioxidant activity levels. The results indicated that HSD induced production performance reduction, immunosuppression, and imbalance redox status, along with elevation in inflammation and stress markers levels. Breast meat weight and yield were reduced in the HSD groups by 9 and 1%, respectively, compared to LSD groups. However, CC supplementation to HSD birds was able to slightly improve daily weight gain, body weight gain, and breast weight, showing no significant difference compared to the LSD-CC group, and significantly increased breast yield. Furthermore, CC supplementation significantly reduced inflammatory cytokines and stress markers levels. Under HSD, both cell-mediated and humeral immune responses were elevated with CC supplementation compared with the non-supplemented group. It can be concluded that HSD is a detrimental factor in the commercial poultry industry, which generates oxidative and inflammatory responses and, subsequently, immunosuppression and impaired performance. Nevertheless, dietary CC supplementation can be used as a natural antioxidant source to mitigate the negative impacts of HSD on broilers' production performance, as well as physiological competency.

Keywords: colocynth seed; broilers performance; meat quality; redox status; immune response; inflammatory cytokines; stocking density

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1. Introduction

The sustainable development goals consist of ending hunger, achieving food security, and promoting sustainable agriculture. The perception of the intensive poultry production system is employed to achieve greater productivity per area, attaining the highest profitability. Admittedly, high stocking density (HSD) is an inevitable practice to accomplish this goal. The official journal of the European Union defined stocking density as "the total live weight of chickens which are present in a house at the same time per square meter of useable area". Thus, increasing stocking density is applied to increase the profitability of the square meter. Nevertheless, there are thoughtful concerns about compromising

broilers' welfare, as well as productivity, when applying HSD. A decade ago, Verspecht et al. [1] stated that maintaining profitability demands a stocking density of approximately 46 kg/m^2. Recently, numerous challenges have faced the commercial broiler production system regarding inputs availability and costs, as well as consumer demand and sales in response to global crises such as the COVID-19 pandemic [2–4] and climate change [5,6], as well as the ongoing conflicts. These production-challenging conditions justify the breeder urge to increase stocking density in order to meet the market demands and earn an adequate profit margin for maintaining sustainable poultry production.

There is an ongoing dispute about the influence of high stocking density (HSD) on broilers' welfare and production performance. Stocking density elevating from 28 to 42 kg/m^2 was reported to negatively impact broiler weight gain and intake, provoking liver injury and elevating body surface temperature [7]. Further, a stocking density of 20 broilers/m^2 adversely influences feed consumption and daily weight increase, generating oxidative damage and an increased mortality rate [8,9]. Additionally, HSD (18 birds/m^2) exhibited a negative impact on broiler's growth performance, wing feathering score, and footpad dermatitis score compared with lowered stocking density (12 birds/m^2) [10]. Under heat stress conditions, elevating the stocking density from 16 and 18 birds/m^2 to 23 and 26 birds/m^2 induced oxidative stress response and reduced the broiler's meat quality [11]. Furthermore, regarding high stocking density (17 birds/m^2), Guardia et al. [12] linked the impairment of broilers' production performance (i.e., the ratio of the feed conversion and daily weight increase) to the changes in the digestive microbiota fingerprint throughout the episode of 32–39 days of age. Bilal et al. [13] indicated that the adverse consequences of HSD not only included growth performance reduction with abnormal gait scores, but also showed immunosuppression and decreased profitability. Conversely, from the veterinary point of view and with the use of the annual mortality, rate of feed conversion, and antimicrobial usage in broiler farms with high (39 kg/m^2) or low (33 kg/m^2) stocking densities, Tarakdjian et al. [14] suggested that stocking density exhibits a minor role in broiler health and welfare. Interestingly, Bergeron et al. [15] stated that daily weight gain is positively linked to stocking density under commercial broiler systems. Furthermore, Nasr et al. [16] reported no difference between low (14 birds/m^2) and medium (18 birds/m^2) stocking densities on carcass features, oxidative stress markers, growth performance, and quality of meat in two varied broiler breeds. However, when stocking density increased to 20 birds/m^2, they reported significant negative impacts on the studied parameters. Generally, in order to alleviate the detrimental effect of oxidative stress generated under high stocking density on broilers' production and physiological performances, different dietary strategies were suggested, including different dietary feed additives (e.g., probiotics, prebiotics, synbiotics, vitamins, and plant products) [17].

Colocynth (*Citrullus colocynthis*) accounts for an herbal plant conventionally utilized in folk medicine. The highest antioxidant activities were found in colocynth seeds (CC), with a documented presence of 28 significant antioxidant active metabolites compared to other fruit parts (i.e., rinds and pulps) [18]. Our research group identified the CC seed phenolic compounds and fatty acids profiles [19]. The data revealed the presence of 18 polyphenol compounds with high concentrations of chlorogenic acid, p-hydroxy benzoic acid, and rosmarinic acid, among other antioxidant and anti-inflammatory bioactive phytochemical compounds. In addition, Bourhia et al. [20] reported that CC extract possesses antioxidant, antibacterial, and anticancer bioactivities. Furthermore, CC extracts exhibit potential pharmacological activities, which include analgesic and anti-inflammation [21–23]. Dietary CC supplementation was found to be potent immunomodulation and natural growth promoter agent for broilers reared under heat stress-induced-oxidative stress conditions [24]. Moreover, dietary CC supplementation was found to be an effective strategy to reduce the consequences of severe oxidative stress induced via paraquat on laying hens' production and immune responses [19]. Thus, we aimed to identify the HSD's negative consequences on broilers' immune response, inflammation, and redox status. Moreover, we investigated

the antioxidant, immune-modulation, and anti-inflammation potentials of dietary CC supplementation to sustain broiler performance under HSD rearing conditions.

2. Materials and Methods

2.1. Birds Management and Experimental Design

Six hundred and forty-eight Cobb 500 male broiler chickens, 21 days old, have been recruited from a commercial broiler flock. The birds were allocated to a 2 × 2 factorial arrangement according to the stocking density (low (LSD; 9 birds/m^2) or high (HSD; 18 birds/m^2)) and colocynth (*C. colocynthis*) seed supplementation (CC; 0 or 1 g/kg feed) as the primary factors. The experimental groups were as follows: LSD without CC supplementation (LSD − CC), LSD with CC supplementation (LSD + CC), HSD without CC supplementation (HSD − CC), and HSD with CC supplementation (HSD + CC). Each experimental group comprises six replicate pens containing 18 birds for LSD groups and 36 birds for HSD groups. During the experiment period, water and feed were provided *ad libitum*. The basal diet of the experiment was prepared to meet the bird's requirement following the Cobb 500 rearing guide (https://www.cobb-vantress.com; accessed on 15 October 2022). The basal diet was corn-soybean meal based diet with 3150 kcal/kg metabolizable energy and 20% crude protein [24]. For CC supplemented groups, colocynth seed was daily grinded to fine powder and was gradually mixed with the basal diet. Birds were housed in 1.6 × 1.6 m pens that contained two feeders and one bell-shaped drinker, with a total mobility space of 2.052 m^2. The brooding temperature has been kept at 22 ± 3 °C during the experimental period (21 to 35 days of age) with a lighting pattern of only 4 hrs dark per day and 20 hrs light. Broilers were reared on floor pens with a deep litter floor brooder consisting of a wood shave (5 cm). Cooling pads and fans were employed to regulate the ambient temperature and ventilation.

2.2. Colocynth Phenolic Compounds Profile

Using high-performance liquid chromatography, colocynth seed phenolic components were identified (HPLC). The approach outlined in [25] was followed in the sample preparation process. HPLC equipment (Agilent 1260 infinity HPLC Series, Agilent, Palo Alto, CA, USA) was used to conduct the test methodology described in Agilent Application Note (No. 5991-3801EN; 2014). The separation was carried out through a Kinetex® 5 m EVO C18 100 (100 4.6 mm) column and an HPLC with quaternary pump (Phenomenex, CA, USA), functioned at 30 °C utilizing a ternary linear elution gradient (HPLC-grade water, 0.2% H_3PO_4 (*v*/*v*), acetonitrile, and methanol). The injection volume attained 20 µL. The phenolic compounds were detected using a variable wavelength detector (VWD) customized at 284 nm. Figure 1 lists the phenolic compounds in colocynth seed identified by HPLC.

2.3. Production Performance Parameters

The preliminary body weight was obtained on the 21st day of the age, whereas the final body weight was obtained on the 35th day. During these 14 days, feed intake (FI) was reported weekly and feed conversion (FCR) was accordingly estimated.

2.4. Meat Quality

At the end of the experiment period, one chicken from each group replicate was randomly selected, weighted, and slaughtered for breast muscle harvesting. The breast muscle weight was recorded using a sensitive digital balance of 0.1 g, and breast yield percentage was calculated relative to the preslaughter live body weight. The breast muscle color was assessed postmortem applying the CIELAB system using an automated color reader (Konica Minolta, Tokyo, Japan). The breast meat pH was determined at a depth of 1 cm after chilling for 24 h postmortem by a pH meter (Hanna Instruments, Venice, Italy). The cooking loss of muscle samples were assessed at 24 h postmortem as described by Lu et al. [26].

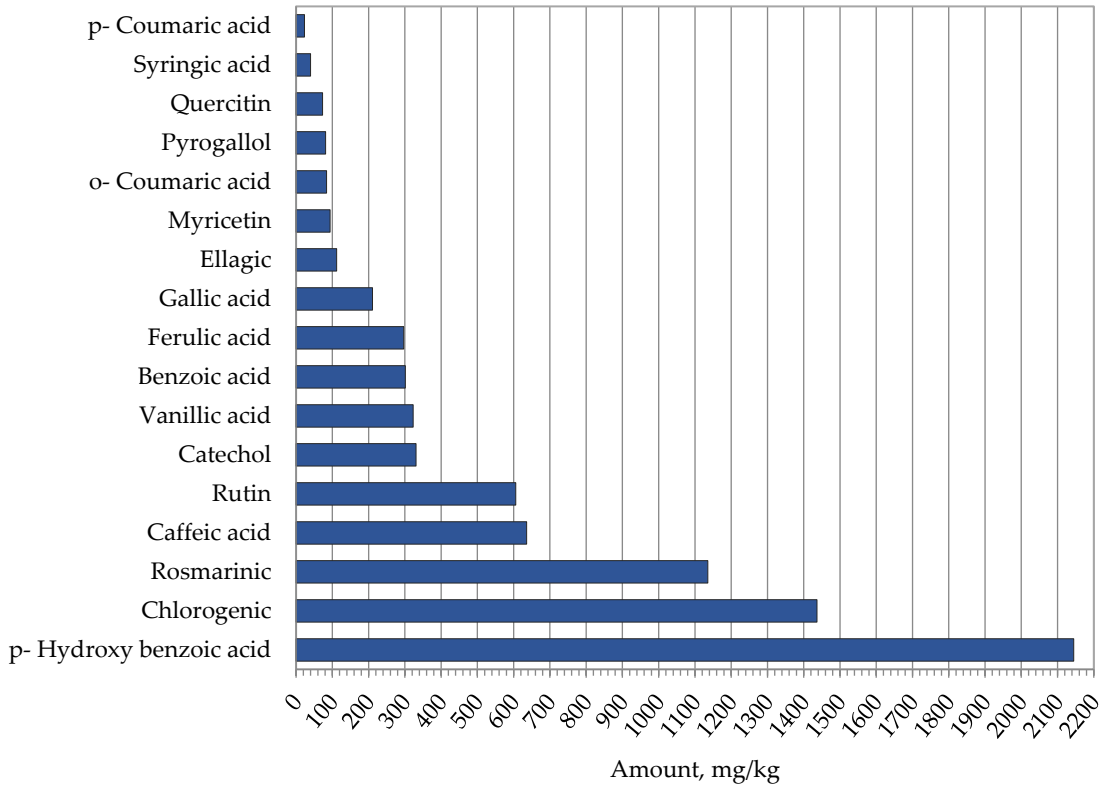

Figure 1. Phenolic acids profile of colocynth seed. Data are introduced, on a dry matter basis, as mg phenolic compound per each kg of colocynth seed.

2.5. Blood Sampling and Preparation

Samples of blood were obtained from each experimental group (n = 6, one sample per replicate) at the end of the 5th week of the age. Samples were left for 10 min in a centrifuge operated at 2000× *g* and 4 °C. Serum was then isolated and maintained at −20 °C for the analysis of immunoglobulins, inflammatory cytokines, and antioxidant activity.

2.6. Stress Markers Quantification

The H/L ratio was manually estimated in whole blood sample (n = 6, one sample per each group replicate) depending on the method given by Kamel et al. [27]. The H/L ratios were computed after the differential counts of a total of 200 leukocytes in six samples per experimental group utilizing a light microscope. Serum corticosterone hormone was assessed via chicken-specific quantitative competitive ELISA kits (cat#: MBS701668; MyBioSource, San Diego, CA, USA). According to the manufacturer, the assay sensitivity is <0.5 ng/mL with intra- and inter-assay precision of <8 and <10%, respectively. Serum malondialdehyde (MDA) level was estimated following the thiobarbituric acid technique by means of a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.7. Serum Antioxidant Activity

The quantification of superoxide dismutase (SOD), total antioxidant capacity (TAC), and catalase (CAT) activities were executed in serum samples (n = 6, one sample per each group replicate) through colorimetric kits (cat#: MBS2563691, MBS2540515, and MBS2540413, respectively; MyBioSource, San Diego, CA, USA). According to the manufacturer, the kit sensitivity, intra-assay precision, and inter-assay precision for SOD were 1.35 U/mL, 5.1% and 9.6%; for TAC, were 0.62 U/mL, 2.7% and 8.2%; and for CAT, were 0.27 U/mL, 3.1% and 5.1%, respectively.

2.8. Immune and Inflamation Response Parameters

2.8.1. Leucocytes Cell Count and Viability Measures

Total white blood cells (TWBCs) counts were executed manually in six complete blood samples per each experimental group (n = 6, one sample per group replicate) following Gehad et al. [28]. Whereas leucocyte cell viability (n = 6; one sample per replicate) was measured as Abbas et al. [29] described.

2.8.2. Immunoglobulins Concentration

The serum immunoglobulin (Ig) A, G, and M concentrations were measured (n = 6, one sample per each group replicate) using chicken ELISA kits (cat#: CSB-E11232Ch, CSB-EQ027259CH, CSB-E16200C, respectively; Cusabio Biotech Co., Ltd., Wuhan, China). According to the manufacturer of the kit, the intra- and inter-assay precision for IgA were <8 and <10%; for IgG, were <15 and <15%; and for IgM, were <8 and <10%, respectively.

2.8.3. The Humoral-Mediated Immune Response

Sheep red blood cells (SRBCs) antibody titer was estimated to assess the humoral-mediated immune response. At the age of 28 days, 12 chickens were randomly allocated from each examined group (two birds per replicate) and injected intravenously with 1 mL of 5% SRBCs saline suspension. After seven days, post-SRBCs injection, blood samples were taken, and the SRBCs antibody production was assessed through the micro-hemagglutination method [30]. The values of SRBCs antibody production were expressed when visible agglutination was observed as log2 of the reciprocal of the highest dilution.

2.8.4. The Cell-Mediated Immune Response

Wattle thickness, developed due to injection of phytohemagglutinin-P (PHA) antigen, was accomplished to examine the cell-mediated immune response, as described by Edelman et al. [31]. In brief, six birds per each examined group (one bird per replicate) were randomly assigned and injected with 50 µL of PHA solution into the center of the wattle. The wattle thickness was then calculated by subtracting the thickness 24 h after PHA injection from the thickness before injection using Schnelltester automatic caliper.

2.8.5. Inflammatory Cytokines

Blood serum levels of interleukins IL-6, IL-10, IL-1β, and tumor necrosis factor-alpha (TNF-α) were estimated (n = 6, one sample per each group replicate) by chicken-specific ELISA kits (cat#: MBS2021018, MBS701683, MBS2024496 and MBS2031870, respectively; MyBioSource, San Diego, CA, USA). According to the manufacturer, the kit sensitivity, intra-assay precision, and inter-assay precision for IL-6 were <5.6 pg/mL, <10% and <12%; for IL-10, were <0.5 pg/mL, <15% and <15%; for IL-1β, were <2.9 pg/mL, <10% and <12%; and for TNF-α, were <3.7 pg/mL, <10% and <12%, respectively.

2.9. Statistical Analysis

Data were analyzed through the two-way analysis of variance according to the general linear model (GLM) of the SAS software package. The statistical model includes the type of stocking density (SD), the CC supplementation, and their interaction as the main effects. When the interaction effect was significant, the Duncan post-hoc test was accomplished in order to determine the significance amongst the experimental groups at $p < 0.05$. Data results were presented as means ± SEM.

3. Results

3.1. Production Performance

The production performance of broilers reared under low or high stocking density with or without CC supplementation is presented in Table 1. Irrespective of CC supplementation, the results revealed a considerable decrease in average daily gain and final body weight in the HSD group relative to the LSD group. Furthermore, the feed conversion ratio was

reduced in the HSD groups relative to the LSD groups, with no impact of HSD on average daily feed intake. However, the supplementation of CC substantially lessened the negative influence of HSD on broilers' body weight and body weight gain, yet it had no influence on the conversion ratio compared to the non-supplemented LSD-CC group. No interaction between SD and CC supplementation was detected.

Table 1. Production performance of broiler chickens reared under low (LSD, 9 birds/m^2) or high (HSD, 18 birds/m^2) stocking density and feed diet with or without colocynth seed supplementation (CC, 1 g/kg feed).

Parameters	LSD		HSD		SEM	*p*-Value		
	−CC	+CC	−CC	+CC		SD	CC	SD × CC
BW3wks, g	744	745	756	753	6.51	0.495	0.929	0.882
BW5wk, g	1894	1987	1785	1804	22.8	0.0003	0.109	0.284
ADG, g	82.1	88.7	73.5	75.1	1.69	0.0002	0.109	0.322
ADFI, g	125	133	121	121	2.17	0.068	0.422	0.368
FCR	1.53	1.49	1.65	1.61	0.01	<0.0001	0.018	0.859

Means having various superscripts significantly differ ($p < 0.05$). BW3wks: body weight after 3 weeks of age; BW5wks: body weight after 5 weeks of age; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio.

3.2. Meat Quality

The breast meat quality parameters of broilers reared at various stocking densities with CC supplementation are displayed in Table 2. The HSD-CC group showed a significant reduction in breast weight and breast yield by 8 and 2%, respectively, relative to the LSD-CC group. Nevertheless, meat color and cooking loss percentage were not affected by the stocking density. Furthermore, the CC supplementation to broiler chickens under HSD was able to improve the breast weight and yield. Moreover, the meat pH was significantly elevated in the HSD and non-supplemented CC groups compared to the LSD- and CC-supplemented groups.

Table 2. Breast meat quality parameters of broiler chickens reared under low (LSD, 9 birds/m^2) or high (HSD, 18 birds/m^2) stocking density and feed diet with or without colocynth seed supplementation (CC, 1 g/kg feed).

Parameters	LSD		HSD		SEM	*p*-Value		
	−CC	+CC	−CC	+CC		SD	CC	SD × CC
Breast weight, g	169	180	156	162	2.21	<0.0001	0.009	0.374
Breast yield, %	8.94	9.05	8.77	8.97	0.03	0.037	0.011	0.402
pH, 24 h	5.77	5.82	5.77	5.78	0.01	0.026	0.002	0.074
L*	49.4	49.7	49.9	49.5	0.22	0.6800	0.8716	0.422
a*	4.39	4.54	4.40	4.50	0.04	0.822	0.131	0.740
b*	9.28	9.73	10.13	10.04	0.18	0.119	0.618	0.453
Cooking loss, %	20.7	20.9	21.0	21.0	0.22	0.671	0.848	0.876

Means having various superscripts significantly differ ($p < 0.05$). L*: lightness; a*: redness; b*: yellowness.

3.3. Stress Markers and Antioxidant Status

The stress markers and antioxidant enzyme activity of broilers reared at different stocking densities and with or without CC supplementation are presented in Table 3. Birds under HSD significantly exhibited high levels of stress markers (i.e., corticosterone, H/L ratio, and MDA), low antioxidant enzymes activity (i.e., catalase and SOD), and a reduction in total antioxidant activity. The H/L ratio, concentration of corticosterone, and MDA level were significantly elevated by 1.7-, 1.8-, and 2.2-fold in the HSD-CC group relative to the LSD-CC group, respectively. Additionally, significant 1.7-, 1.4-, and 1.4-fold reductions were noticed in SOD, catalase, and T-AOC activities, respectively, in the HSD-CC group relative

to the LSD-CC group. Under HSD rearing conditions, CC supplementation significantly reduced the H/L ratio and increased the SOD activity. Furthermore, under LSD-rearing conditions, CC supplementation significantly increased the T-AOC activity.

Table 3. Stress markers and antioxidant enzymes activity of broiler chickens reared under low (LSD, 9 birds/m^2) or high (HSD, 18 birds/m^2) stocking density and feed diet with or without colocynth seed supplementation (CC, 1 g/kg feed).

Parameters	LSD		HSD		SEM	*p*-Value		
	−CC	+CC	−CC	+CC		SD	CC	SD × CC
CORT, pg/mL	4.61	4.03	8.29	7.42	0.40	<0.0001	0.029	0.653
H/L Ratio	0.37 ^{c}	0.35 ^{c}	0.63 ^{a}	0.50 ^{b}	0.03	<0.0001	0.006	0.046
MDA, µM/mL	1.98	1.95	4.35	3.58	0.20	<0.0001	0.063	0.084
SOD, U/ml	5.05	5.15	2.98	3.70	0.22	<0.0001	0.067	0.161
Catalase, U/mL	0.83	0.91	0.59	0.67	0.03	<0.0001	0.009	1.000
T-AOC, U/mL	8.34 ^{b}	10.38 ^{a}	5.88 ^{c}	6.25 ^{c}	0.40	<0.0001	0.0006	0.011

Means having various superscripts significantly differ ($p < 0.05$). CORT: corticosterone; H/L ratio: heterophil-to-lymphocyte ratio; MDA: malondialdehyde; SOD: superoxide dismutase; T-AOC: total antioxidant capacity.

3.4. Inflammation Markers

The pro-inflammatory cytokines concentrations of broilers reared under various stocking densities and with or without CC supplementation are presented in Table 4. The results indicated the induction of inflammation in response to the HSD condition. The fold increase in TNF-α, IL-6, IL-10, and IL-1β concentrations for the HSD-CC group compared with the LSD-CC group were 1.7-, 3.2-, 3.4-, and 2.1-fold, respectively. Additionally, CC supplementation to the HSD group exhibited an anti-inflammation effect and significantly decreased the pro-inflammatory cytokine concentrations compared to the non-supplemented HSD group by 16, 21, 28, and 18% for TNF-α, IL-6, IL-10, and IL-1β, respectively. A significant interaction was detected between SD and CC supplementation for cytokine levels.

Table 4. Serum pro-Inflammatory cytokines concentration of broiler chickens reared under low (LSD, 9 birds/m^2) or high (HSD, 18 birds/m^2) stocking density and feed diet with or without colocynth seed supplementation (CC, 1 g/kg feed).

Parameters	LSD		HSD		SEM	*p*-Value		
	−CC	+CC	−CC	+CC		SD	CC	SD × CC
TNF-α, pg/mL	90.8 ^{c}	84.8 ^{c}	155.7 ^{a}	130.0 ^{b}	6.40	<0.0001	0.002	0.039
IL-6, pg/mL	3.27 ^{c}	2.90 ^{c}	10.53 ^{a}	8.30 ^{b}	0.71	<0.0001	0.005	0.035
IL-10, pg/mL	2.63 ^{c}	2.38 ^{c}	9.03 ^{a}	6.53 ^{b}	0.59	<0.0001	<0.0001	0.001
IL-1β pg/mL	271 ^{c}	243 ^{c}	556 ^{a}	453 ^{b}	28.3	<0.0001	0.002	0.049

Means having various superscripts significantly differ ($p < 0.05$). TNF-α: tumor necrosis factor alpha; IL-6: interleukin 6; IL-10: interleukin 10; and IL-1β: interleukin1β.

3.5. Immune Responses

The humoral and cell-mediated immune responses of broilers reared at various stocking densities with CC supplementation are displayed in Table 5. In general, all the experimentally-considered immune-response parameters were negatively affected by HSD compared to LSD. The leucocyte cell count (TWBCs) was reduced by 31% and 22%, whereas the leucocyte cell viability percentage was reduced by 16% and 12% in the HSD-CC and HDS + CC groups relative to the LSD-CC group, respectively. Furthermore, the HSD − CC group manifested serum immunoglobulins concentration reduction by 1.7-, 1.9-, and 1.7-fold for IgA, IgG, and IgM, respectively, relative to the LSD − CC group. Additionally, the antibody production against SRBC was significantly reduced by 1.6-fold. The wattle cell-mediated immune response to PHA antigen injection was negatively affected in the HSD − CC group, showing 37% wattle thickness reduction relative to the LSD − CC group.

However, the CC supplementation to HSD birds considerably improved the serum immunoglobulins concentrations, SRBC antibody production, and cell-mediated response to PHA-wattle injection compared to the non-supplemented HSD group. It is worth noticing that, under LSD rearing conditions, CC supplementation elevated the IgA and IgM serum concentrations, and improved the birds' cell-mediated response to PHA-wattle injection. A significant interaction between SD and CC supplementation was only observed for the SRBC antibody parameter.

Table 5. Immune response parameters of broiler chickens reared at low (LSD, 9 birds/m^2) or high (HSD, 18 birds/m^2) stocking density and feed diet with or without colocynth seed supplementation (CC, 1 g/kg feed).

Parameters	LSD		HSD		SEM	*p*-Value		
	−CC	+CC	−CC	+CC		SD	CC	SD × CC
TWBCs, $\times 10^3$/mL	45.4	48.9	31.1	35.3	1.67	<0.0001	0.025	0.825
LCV, %	101.7	107.7	85.3	89.0	2.14	<0.0001	0.034	0.590
IgA, µg/mL	62.7	71.2	36.8	46.8	3.01	<0.0001	0.001	0.758
IgG, µg/mL	137.2	151.7	72.8	87.0	7.55	<0.0001	0.043	0.980
IgM, µg/mL	299	347	172	242	15.00	<0.0001	0.0003	0.424
SRBC-Ab, log2	6.83 ^{ab}	7.17 ^{a}	4.17 ^{c}	6.00 ^{b}	0.28	<0.0001	0.0015	0.020
Wattle thickness, mm	0.35	0.39	0.22	0.28	0.01	<0.0001	<0.0001	0.399

Means having various superscripts significantly differ ($p < 0.05$). TWBC: total white blood cells; LCV: leucocyte cell viability; IgA: immunoglobulin A; IgG: immunoglobulin G; IgM: immunoglobulin M; SRBC-Ab: sheep red blood cells antibody.

4. Discussion

The stocking density elevation was proven to have massive negative impacts, not only on production performance, but also on birds' physiological systems and meat quality. The HSD employed in the current investigation was 18 birds/m^2 with an actual final weight of 32 kg/m^2. Broiler production performance was significantly reduced, in terms of body weight and ADG, and FCR was impaired under the HSD. Several researchers have reported an HSD negative consequence on broiler production performance (i.e., feed conversion, feed intake, and body weight gain) [32–36]. However, other researchers reported no effect [15]. These controversial results can be justified by the influence of other rearing conditions, such as ventilation and age, on performance. Under the current study HSD condition, CC supplementation significantly improved BW and ADG with no effect on feed intake and FCR. Such CC supplementation's positive effect is likely attributed to its antioxidant activities, which helped in mitigating the negative consequences of HSD-induced-oxidative-stress on broiler performance [19,24]. In the present study, breast meat weight and yield were reduced in the HSD-CC group with no effect on meat color and cooking loss. Nasr et al. [16] reported a reduction in breast yield and meat quality parameters associated with HSD in Arbor acres and Ross-308 broiler breeds. By contrast, other researchers indicated no effect of different stocking densities (ranged from 12.6 up to 40.91 kg/m^2) on meat quality parameters [11,37,38].

The stress markers of broilers reared under HSD showed considerably higher values than LSD. These findings agree with those presented by other researchers who documented an associated increase in corticosterone concentration [39,40] and H/L ratio [37,41,42] along with increasing stocking density. The corresponding results of antioxidant enzyme activity drop, as well as an increase in the level of lipid peroxidation metabolite observed under the current HSD rearing conditions, demonstrate a state of oxidative stress induction. Son et al. [11] found an increase in liver MDA concentration, as well as a reduction in blood SOD activity, in broilers reared under HSD. Miao et al. [8] also reported that plasma MDA level was increased, whereas the T-AOC was decreased with HSD (20 birds/m^2) compared with LSD (14 birds/m^2).

On one hand, there is a close interrelation between oxidative stress, immune response, and inflammation [43]. Cytokines, which represent the regulators and mediators of host immune responses, include pro-inflammatory cytokines (e.g., IL-6, TNF-α, and IL-1β) that are involved in the induction of fever, inflammation, and tissue destruction [44,45]. The elevated pro-inflammatory cytokines levels are one of the significant negative impacts of HSD under the current study condition and can directly be connected to the detected immunosuppression state. The anti-inflammation property of CC has been demonstrated by several researchers [21–23]. Thus, the CC supplementation to HSD birds significantly reduced pro-inflammatory cytokines levels, reflecting its anti-inflammation properties.

On the other hand, the present study supported the likelihood that HSD caused immunosuppression, with the observed decrease in both humoral (i.e., immunoglobulins and SRBC-AB levels) and cell-mediated (i.e., PHA-injection immune response) immune responses. Li et al. [46] reported that HSD induced physiological and oxidative stresses with a significant reduction in serum immunoglobulin (IgA and IgG) concentration. Moreover, Sevim et al. [47] reported a significant increase in lymphocyte DNA damage for broilers reared under HSD (18 birds/m^2). Moreover, a high corticosterone level was previously demonstrated to have an immunosuppression effect [48]. In the current experiment, the corticosterone level was 61 to 80% higher in the HSD groups relative to the LSD-CC group, which might partially justify the detected immunosuppression. Furthermore, regardless of the stressor type, oxidative stress generation was reported to induce immunosuppression in broilers [27,49,50]. Moreover, Xu et al. [45] concluded that the immune response was mediated via the level of the inflammatory cytokine due to infectious bursal disease virus (IBDV) infection in chickens. Thus, the oxidative stress generation and the high corticosterone and pro-inflammatory cytokines concentration induced by HSD in the current study could be responsible for the detected immunosuppression. However, the reported immunomodulation and antioxidant bioactive properties of CC contributed to alleviating the negative impacts of HSD, partially reducing stress markers levels, and improving broiler immune response [19,24].

5. Conclusions

Stocking density is still a controversial concern for the sustainability of the commercial poultry industry. Under intensive commercial broiler production, increasing stocking density is an inevitable stressor. The present study highlighted the HSD negative impact on broilers' production performance with a new insight into birds' immune response, inflammation induction, and antioxidant status. The evidence from this study revealed profound immunosuppression, inflammation, and imbalance redox status when rearing chickens under the presence stocking conditions. However, the supplementation of CC as a natural antioxidant and anti-inflammation agent by 1 g/kg feed revealed positive mitigation of the immune response and inflammatory cytokine levels. Further works are needed to be done to alleviate the negative impacts of HSD under commercial production conditions. Nevertheless, it can be concluded that natural antioxidant and anti-inflammatory products, such as CC seed, can effectively improve the physiological mechanisms and sustain broiler production performance under HSD rearing conditions.

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