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Aspirin Eugenol Ester Ameliorates Hypothalamic Neuroinflammation and Improves Growth Performance in High-Density-Raised Broilers

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Abstract

High-stocking-density (HD) environments can trigger systemic inflammatory responses, consequently impairing broiler growth. Given the broad anti-inflammatory properties of aspirin eugenol ester (AEE), this study investigated the effects of AEE supplementation on growth performance, immune organ indices, serum immunoglobulin levels, and hypothalamic inflammation-related markers in HD broilers. A total of 528 one-day-old male Arbor Acres (AA) broilers were randomly assigned to four groups: ND, HD, ND-AEE, and HD-AEE (ND, 14 birds/m^2; HD, 22 birds/m^2), with six replicate cages per treatment group over a 42-day experimental period. The results revealed that AEE significantly improved the growth performance of HD broilers. Immune organ indices, serum immunoglobulin levels, and the expression of spleen inflammatory factors was associated with the organismal inflammatory response, which manifested primarily during the late growth phase. On Day 35, AEE significantly suppressed ($p < 0.05$) the relative mRNA expression of p21-activated kinase 1 (PAK1) in the hypothalamus of HD broilers. On Day 42, AEE significantly reduced the relative mRNA expression of PAK1, p38 mitogen-activated protein kinase (p38MAPK), cyclooxygenase-2 (COX-2), prostaglandin E synthase 1 (mPGES-1), and interleukin-1β (IL-1β) ($p < 0.05$), while significantly elevating the relative mRNA expression of growth hormone-releasing hormone (GHRH) ($p < 0.05$). Collectively, these findings demonstrate that AEE mitigates high-density rearing-induced hypothalamic inflammation and is associated with downregulated mRNA expression of PAK1 and its downstream targets in the p38MAPK/COX-2 axis. This gene expression profile correlates with improved growth and immune function in high-density-stressed broilers, suggesting a potential regulatory link that requires further validation at the protein and functional levels.

Keywords: high stocking density; broiler; aspirin eugenol ester; hypothalamus; RNA-seq

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1. Introduction

In recent years, global demand for chicken meat has steadily risen. While the broiler industry has embraced intensive farming to boost production efficiency, excessively high stocking densities have triggered multiple health problems in broilers which ultimately undermine economic returns [1–3]. The extreme stocking density of 39 kg/m^2 or 16 birds/m^2 remains the prevailing industry standard [4], yet breeding enterprises often exceed this threshold in pursuit of greater profits. Most studies have indicated that high stocking density (HD) significantly impairs broiler performance, curbs feed consumption and body weight gain, elevates the feed conversion ratio (FCR), and hinders overall growth [5]. HD is a well-documented trigger for stress in broilers. Under overcrowded rearing conditions, broilers face severe spatial limitations and intense competition, resulting in marked stress responses and significantly elevated plasma corticosterone levels [6]. Furthermore, multiple stressors simultaneously challenge the physiological systems of broilers in high-density environments, often triggering excessive inflammatory responses. Numerous studies have confirmed elevated serum concentrations of inflammatory factors in densely housed birds [7]. These vital signaling molecules, released by the body in response to infection or injury, can severely impair health when persistently elevated. Moreover, research has indicated that HD reduces spleen and bursa weights in broilers [8,9], potentially compromising the organism's immune function. Consequently, identifying strategies to effectively mitigate the detrimental effects of HD has become a critical focus within the broiler industry.

The organism's energy balance is orchestrated by the central nervous system, with the hypothalamus serving as a crucial regulatory center controlling diverse physiological activities, such as body temperature, feeding, and energy homeostasis [10]. By integrating appetite-related signals, the hypothalamus directly regulates broiler feeding behavior. Crucially, hypothalamic inflammation is a key disruptor of energy balance [11], typically characterized by the activation of various mediators and pathways, including cytokines [12,13], chemokines [14], signaling molecules, and autophagic processes [15]. Exposure to adverse environments can trigger hypothalamic inflammation and consequently impair appetite [16,17], thus undermining growth and health. Based on these observations, it is hypothesized that the complex and stressful HD environments induce hypothalamic-mediated inflammatory responses, which subsequently impair broiler growth.

Aspirin eugenol ester (AEE) has emerged as a promising novel non-steroidal anti-inflammatory drug in recent years. Synthesized through the condensation of aspirin's free carboxyl group with eugenol's hydroxyl group via an acyl chloride reaction [18], AEE offers significant advantages over its parent compounds. It boasts significantly lower toxicity, a prolonged duration of action, and a notably wider safety margin. Furthermore, AEE delivers sustained anti-inflammatory activity while effectively alleviating common side effects such as gastrointestinal irritation and oxidation susceptibility [19]. Previous studies have demonstrated the anti-inflammatory effects of AEE through downregulation of cyclooxygenase 2 (COX-2) expression [20]; however, its precise mechanism is not completely understood. Intriguingly, dietary AEE supplementation in broilers reared under high-density feeding conditions has been reported to enhance feed intake and daily weight gain, thus improving overall growth performance [21].

In the context of intensive poultry production, HD rearing is a common practice that often induces hypothalamic inflammation and subsequent impairments in growth and immune function—key challenges compromising production efficiency. AEE, a derivative with both anti-inflammatory and antioxidant properties, has emerged as a potential dietary intervention, though its specific regulatory roles in modulating HD-induced hypothalamic inflammation and associated growth perturbations remain poorly characterized. To address

this knowledge gap, the present study aims to investigate the effects of AEE on production performance, hypothalamic inflammatory responses, and growth-related indicators in HD-housed broilers, with a focus on the PAK1/p38MAPK/COX-2 signaling axis—a pathway closely linked to inflammatory cascades and metabolic regulation in the hypothalamus. This investigation is intended to clarify the potential associations between AEE supplementation, hypothalamic gene expression patterns, and amelioration of HD-induced physiological dysfunctions, laying a foundation for further exploration of the underlying mechanisms.

2. Materials and Methods

2.1. Ethical Treatment

Approval for the experimental protocol was granted by the Committee for the Management and Use of Laboratory Animals at Henan University of Science and Technology (AW20602202-1-2). All experimental procedures were conducted adhering to established ethical guidelines for animal research.

2.2. Animals and Experimental Design

In this experiment, conducted in October 2024, 528 one-day-old healthy Arbor Acres (AA) male broilers with similar body weights were rigorously selected and randomly assigned to one of four distinct experimental groups: the normal density group (ND, 14 broilers/m^2), the high-density group (HD, 22 broilers/m^2), the normal density + 0.01% AEE group (ND-AEE), and the high-density + 0.01% AEE group (HD-AEE). To maintain constant stocking density (a core experimental factor) throughout the 42-day trial and avoid confounding effects from bird removal for sampling, the experiment adopted a dual-cohort design: a main experimental cohort for growth performance monitoring, and a dedicated sampling cohort for tissue/serum/RNA-seq collection. Initially, the cage size was established as 1 m^2 per cage (length: 1 m × width: 1 m), corresponding to the precise dimensions employed in the experimental protocol. For the main experimental cohort, utilized exclusively for growth performance monitoring without bird removal during the trial, the bird count per cage was meticulously calculated to achieve the targeted stocking densities. Specifically, the ND and ND-AEE groups each contained 14 birds per cage (14 birds/m^2 × 1 m^2 = 14 birds/cage), with six replicate cages per group (14 birds/cage × 6 replicates = 84 birds per group). Similarly, the HD and HD-AEE groups each comprised 22 birds per cage (22 birds/m^2 × 1 m^2 = 22 birds/cage), also with six replicate cages per group (22 birds/cage × 6 replicates = 132 birds per group). Consequently, the total bird count in the main cohort was 84 (ND) + 132 (HD) + 84 (ND-AEE) + 132 (HD-AEE) = 432 birds. For the dedicated sampling cohort, allocated to collect biological samples without disrupting the main cohort's density, 24 birds were assigned per group (6 replicate cages × 4 sampling time points × 1 bird sampled per time point = 24 birds per group), across four groups (24 birds/group × 4 groups = 96 birds in the sampling cohort). These birds were housed in parallel cages of identical 1 m^2 size, matching the stocking density and diet of their corresponding main cohort groups. The overall experimental bird total thus comprised the sum of the main and sampling cohorts: 432 (main cohort) + 96 (sampling cohort) = 528 birds. This configuration ensured that the main growth performance cages maintained constant stocking densities (14 or 22 birds/m^2) throughout the experiment, eliminating confounding effects associated with progressive bird reduction. The experimental birds were obtained from Henan Quan Da Poultry Breeding Co., Ltd. (Hebi, China).

All broilers originated from the same breeder flock and hatching week to minimize inter-individual variability in serological and growth traits. The rearing environment was strictly controlled: temperature was maintained at 33–35 °C (Days 1–3), 30–32 °C (Days 4–7), 27–29 °C (Days 8–14), 24–26 °C (Days 15–21), and 22–24 °C (Days 22–42); humidity was kept

at 55–65% throughout the experiment; mechanical ventilation was used to ensure an air exchange rate ≥0.5 m^3/(bird·h) and ammonia concentration < 15 ppm (monitored daily). The lighting program was set as 23 h light (L):1 h dark (D) from Days 1–7 and 18 h L:6 h D from Days 8–42 (light intensity: 20–30 lux at bird level). All broilers received the same vaccination schedule: Newcastle disease vaccine (intranasal, Day 7), infectious bronchitis vaccine (intranasal, Day 7), and infectious bursal disease vaccine (drinking water, Day 14). Monensin sodium (120 mg/kg) was included in the basal diet from Days 1–42 as a coccidiostat. During the initial phase (Days 1–21), the birds were provided with a starter basal diet, followed by a grower basal diet during the subsequent phase (Days 22–42). The specific composition and nutritional profile of these basal diets are presented in Table 1. Throughout the experimental period, the diet was supplemented with AEE, procured from the Institute of Animal Husbandry and Veterinary Medicine of the Chinese Academy of Agricultural Sciences (CAAS, Lanzhou, China). AEE was first mixed with 5 kg of basal diet to form a premix (homogenized for 15 min using a twin-screw mixer), then blended with the remaining basal diet (total mixing time: 30 min) to ensure uniform distribution (coefficient of variation < 5%). Feed and fresh water were provided ad libitum throughout the experiment, with feed troughs and waterers checked twice daily to ensure adequate availability. The inclusion level of 0.01% AEE was selected based on prior research by [21], which demonstrated that 0.01% AEE significantly increased the average daily weight gain of HD broilers by 8% and reduced the expression of the intestinal inflammatory factor IL-1β by more than 13%. Therefore, this dosage was adopted as the experimental intervention level in this study.

Table 1. Composition and nutrient content of broiler basal diets.

Ingredients (%)	Starter Phase (1–21 d)	Grower Phase (22–42 d)
Corn	52.79	57.78
Soybean meal	36.89	30
Soybean oil	4	4
Wheat bran	2	2
Calcium biphosphate	1.912	1.623
Talcum powder	1.222	1.171
NaCl	0.3	0.3
Choline chloride	0.3	0.26
DL-methionine	0.265	0.106
Trace element premix [1]	0.2	0.2
L-lysine	0.038	0.045
Vitamin premix [2]	0.03	0.03
Zea gluten meal	0	2.43
Metabolic energy (MJ/kg)	12.40	13.0
Crude protein	21.18	19.84
Lysine	1.14	1.05
Methionine	0.49	0.48
Calcium	1.02	0.85
Available P	0.45	0.42
Total P	0.69	0.63
Threonine	0.77	0.22

[1] Trace element premix provided per kg of the base diet: iron, 80 mg; copper, 10 mg; zinc, 70 mg; iodine, 0.5 mg; manganese, 80 mg; selenium, 0.3 mg. [2] Vitamin premix provided per kg of the base diet: Vitamin A, 9500 IU; Vitamin D3, 62.5 ug; Vitamin E, 30 IU; Vitamin K, 32.65 mg; Vitamin B1, 2 mg; Vitamin B, 66 mg; Vitamin B12, 0.025 mg; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; niacin, 50 mg.

2.3. Growth Performance and Sample Collection

Daily feed intake was meticulously monitored throughout the experiment, and birds were weighed at 14, 21, 28, 35, and 42 days of age to determine body weight, body weight

gain, feed intake, and FCR. Mortality was recorded daily; dead birds were weighed, and the cause of death was assessed. No infectious or density-related mortality was observed during the experiment, with mortality rates < 1% across all groups. At 21, 28, 35, and 42 days, one bird per replicate from the dedicated sampling cohort (n = 6 per group per time point) was randomly selected. Based on the body weight distribution within each replicate cage, birds with weights closest to the group mean were selected for sampling, while those deviating by more than ± 5% were excluded to minimize sampling bias. Blood samples collected from the wing vein were centrifuged at 4 °C for 30 min; and the resulting serum layer was harvested, aliquoted, and stored at −20 °C for subsequent biochemical analyses. Following inhalation anesthesia, the hypothalamus was rapidly dissected on an ice-cold platform, immediately flash-frozen in liquid nitrogen, and stored at −80 °C. The spleen, bursa of Fabricius, and thymus were excised, weighed using an analytical balance (Sartorius, Model ME204E, precision: ± 0.0001 g) to calculate the immune organ index, and rinsed with ice-cold saline. The formula for immune organ index calculation was:

Immune organ index (mg/g) = (Organ weight, mg)/(Body weight of the bird, g).

The spleen was subsequently flash-frozen in liquid nitrogen and stored at −80 °C for subsequent molecular analyses.

2.4. Measurement of Immunoglobulin Concentration

After serum separation, the concentrations of immunoglobulins in the serum were quantified using a competitive enzyme-linked immunosorbent assay kit (Nanjing Jianjian Bioengineering Institute, Nanjing, China). All experimental procedures were performed strictly adhering to the manufacturer's instructions.

2.5. Transcriptome Sequencing and Analyses

An appropriate amount of hypothalamic tissue was homogenized for total RNA isolation using a TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA)—a commercial dedicated extraction kit widely used for high-quality RNA purification from animal tissues. RNA concentration and purity were subsequently quantified via a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA); samples with an OD_{260}/OD_{280} ratio between 1.8 and 2.0 were considered to meet the purity requirement for downstream experiments. RNA integrity was rigorously evaluated by two methods: 1.2% RNA-specific agarose gel electrophoresis (Sigma-Aldrich, St. Louis, MO, USA) (to visualize 28S/18S rRNA bands, with a 28S/18S intensity ratio >1.8 indicating high integrity) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) (RNA Integrity Number, RIN >8.0 was set as the passing standard). Commencing with a minimum of 1 μg of total RNA (meeting the above quality criteria), polyadenylated mRNA was selectively enriched utilizing Oligo(dT) magnetic beads (Thermo Fisher Scientific, Waltham, MA, USA)—a standard method for isolating eukaryotic mRNA based on its poly(A) tail. This purified mRNA was fragmented at 94 °C for 8 min in the presence of 5× fragmentation buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing divalent cations (Mg^{2+}), which induces random cleavage of mRNA into 200–300 bp fragments suitable for library construction. The fragmented mRNA served as the template for first-strand cDNA synthesis primed by random hexamer primers (Thermo Fisher Scientific, Waltham, MA, USA), followed by second-strand cDNA synthesis using DNA polymerase I (New England Biolabs, Ipswich, MA, USA), dNTP mix (Thermo Fisher Scientific, Waltham, MA, USA), and RNase H (Thermo Fisher Scientific, Waltham, MA, USA) to degrade residual mRNA.

After purification with AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA), double-stranded cDNA underwent end repair (using T4 DNA polymerase, Klenow

Fragment, and T4 polynucleotide kinase; New England Biolabs, Ipswich, MA, USA) to generate blunt ends, 3′-end adenylation (via Klenow Fragment (3′→5′ exo$^-$); New England Biolabs, Ipswich, MA, USA) to add a single A base (facilitating adapter ligation), and adapter ligation with indexed Illumina adapters (Illumina, San Diego, CA, USA) using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). AMPure XP magnetic beads were employed again to isolate cDNA fragments ranging from 300 to 400 bp (verified by agarose gel electrophoresis) to ensure uniform fragment size for sequencing. The resulting library underwent PCR amplification for enrichment (using Phusion High-Fidelity DNA Polymerase; New England Biolabs, Ipswich, MA, USA and Illumina PCR primers; Illumina, San Diego, CA, USA) with the following program: 98 °C for 30 s, 12 cycles of 98 °C for 10 s/60 °C for 30 s/72 °C for 30 s, and a final extension at 72 °C for 5 min. A second purification step using AMPure XP beads was performed to remove PCR by-products, yielding the final RNA sequencing library.

Following high-throughput sequencing, raw image files were demultiplexed into FASTQ format and subjected to initial organization. Low-quality sequences, including adapter-contaminated reads at the 3′-end and reads exhibiting an average Phred quality score below 20, were discarded. High-quality reads were aligned to the reference genome using HISAT2, and gene-level read counts were calculated as raw expression values. Expression levels were normalized using Fragments Per Kilobase of transcript per Million mapped reads (FPKM), with genes demonstrating FPKM > 1.0 considered expressed. Differentially expressed genes (DEGs) were identified utilizing the DESeq2 package, under the thresholds $|\log_2(\text{fold change})| \geq 0.585$ (equivalent to 1.5-fold change) and adjusted p-value < 0.05. Functional annotation and pathway enrichment analysis were subsequently conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to elucidate the biological functions and signaling networks of DEGs. For transcriptome sequencing, a total of 24 birds were analyzed at each time point (days 35 and 42), equally distributed among the four groups, with six biological replicates per group (n = 6 per group per time point). RNA-seq samples were exclusively derived from a dedicated sampling cohort, with each replicate originating from a distinct sampling cage to ensure statistical independence. The total number of RNA-seq samples across both time points was 48.

2.6. Quantiative Real-Time PCR (qRT-PCR) Analysis

Total RNA was extracted by homogenizing tissue samples with grinding beads in microcentrifuge tubes using TRizol reagent (Invitrogen Inc., Carlsbad, CA, USA). RNA concentration and purity were measured using a UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). OD260/280 values consistently ranged between 1.8 and 2.0, confirming high purity. Following genomic DNA (gDNA) removal, RNA was reverse-transcribed into complementary DNA (cDNA) using the M-MLV Reverse Transcription Kit (Takara Bio, Shiga, Japan). The mRNA expression of inflammation-related genes was subsequently quantified by real-time PCR using SYBR Green I detection, with GAPDH as the reference gene (primer sequences provided in Table 2). Amplification and dissociation curves were analyzed, and relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

2.7. Statistical Analyses

All data were verified for homogeneity of variance via Levene's test. Two-way ANOVA was performed followed by Duncan's multiple range test for post hoc comparisons. Statistical analyses were conducted using IBM SPSS 26.0 software (SPSS Inc., Chicago, IL, USA). Results are expressed as mean ± standard error of the mean (SEM). Graphical representations of qRT-PCR results were generated using GraphPad Prism 9 software

(GraphPad Software Inc., San Diego, CA, USA). A probability level of $p < 0.05$ was considered statistically significant. For growth performance parameters, including body weight, average daily weight gain, feed intake, and feed conversion ratio, the experimental unit was the main cage ($n = 6$ per group), with data analyzed as cage-level means to prevent pseudoreplication. Fixed factors in the two-way ANOVA comprised 'stocking density' (ND vs. HD), 'AEE supplementation' (with vs. without), and their interaction (density × AEE). For additional parameters, such as immune organ indices, serum immunoglobulin levels, and gene expression, the experimental unit was the individual bird ($n = 6$ per group per time point) derived from a dedicated sampling cohort.

Table 2. Primer sequences used for qRT-PCR analysis.

Gene Name [1]	Primer Sequence (5′→3′)	Accession Number	Product Length (bp)
PAK1	F: TGCCTGCTGCTGCTCTACTA R: GCTGCTGCTGCTGTTCTTCT	NP_001026170.1	152
p38MAPK	F: GCTGCTGCTGCTGCTCTACTA R: GCTGCTGCTGCTGTTCTTCT	NP_989523.1	148
COX-2	F: TGTGCCAGCAGCAAATGCTT R: GGGCTGCTGTTCTTCTGGA	NM_001167719.2	165
mPGES-1	F: AGGCTCAGGAAGAAGGCATT R: CACAGCTCCAAGGAAGAGGA	XM_003730931.3	139
IL-1β	F: TGGGCATCAAGGGCTACAAG R: AGGCGGTAGAAGATGAAGCG	NM_204524.1	156
TNF-α	F: TGCCTGCTGCTGCTCTACTA R: GCTGCTGCTGCTGTTCTTCT	NM_205129	142
GHRH	F: TGATGGACAGCCGTTACCAC R: GCTGGGAAACCCCTCTAACC	XM_015296359.4	135
NPY	F: CCTTCGATGTGGTGATGGGA R: ATGCACTGGGAATGACGCTA	NM_205473.2	161
GAPDH	F: GAACATCATCCCAGCGTCCA R: CGGCAGGTCAGGTCAACAAC	NM_204305.2	145

[1] Accession number refers to Gene bank (NCBI). IL-1β: Interleukin-1β; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-α; IL-10: Interleukin-10; PAK1: p21-activated kinase 1; p38MAPK: p38 mitogen-activated protein kinase; COX-2: Cyclooxygenase-2; mPGES-1: Prostaglandin E synthase 1; GHRH: Growth hormone-releasing hormone.

3. Results

3.1. Growth Performance

The impact of AEE on broiler chicken performance is depicted in Figure 1 and Supplementary Tables S1 and S2. Broilers in the HD group exhibited significantly lower body weights than those in the ND group at 21, 28, and 35 days of age ($p < 0.05$). Supplementing the basal diets of HD broilers with 0.01% AEE significantly increased body weights in the HD-AEE group compared to the HD group at 21 days ($p < 0.05$). The HD-AEE group demonstrated significantly higher weight gain and feed intake compared to the HD group during the 21–28 day period ($p < 0.05$). Furthermore, adding AEE to the HD broiler basal diet resulted in significantly lower FCRs at 21–28, 28–35, and 35–42 days of age compared to the HD group ($p < 0.05$). At 42 days, BW in the HD group remained numerically lower than in the ND group but did not reach statistical significance ($p > 0.05$). No significant differences in growth performance parameters were observed between the ND and ND-AEE groups ($p > 0.05$).

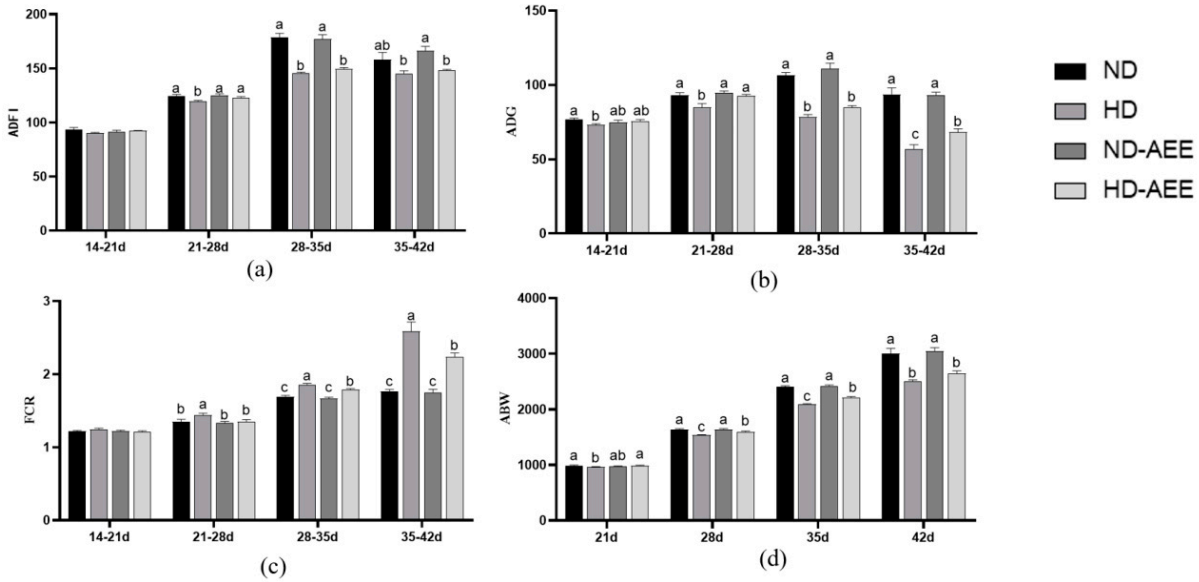

Figure 1. Effect of AEE on growth performance in HD broilers. (**a**) Average daily feed intake; (**b**) Average daily weight gain; (**c**) Average feed conversion ratio (FCR); (**d**) Average body weight. Each vertical bar represents the mean ± SEM (n = 6). Values with different letters are significantly different ($p < 0.05$).

3.2. Immune Organ Index

The impact of AEE on broiler immune organ indicesis depicted in Figure 2 and Supplementary Table S3. Thymus indices showed no significant differences between groups at 21, 28, or 42 days. While the HD group exhibited a decreasing trend in thymus index compared to the ND group at 35 days, this difference failed to reach statistical significance. However, the HD-AEE group showed a significant increase in thymus index relative to the HD group ($p < 0.05$).

Bursa indices revealed no significant differences at 21, 28, or 35 days. By 42 days, the bursal index was significantly lower in the HD group compared to the ND group ($p < 0.05$), while no significant difference emerged between the HD and HD-AEE groups.

Splenic indices displayed no significant differences at 21 days or 42 days. At 28 days, the splenic index was significantly lower in the HD group compared to the ND group ($p < 0.05$), whereas the HD-AEE group demonstrated a significant increase relative to the HD group ($p < 0.05$). Similarly, at 35 days, the splenic index was significantly higher in the HD-AEE group compared to the HD group ($p < 0.05$), with no significant difference observed between the ND and HD groups.

3.3. Serum Immunoglobulin Concentration

The effect of AEE on serum immunoglobulins in broilers is depicted in Figure 3 and Supplementary Table S4. Compared to the ND group, HD group exhibited significantly reduced ($p < 0.05$) serum IgG content at 42 days. Furthermore, adding AEE to the basal diet significantly increased ($p < 0.05$) serum IgM content at 35 days and IgG content at 42 days compared to the HD group. Serum CORT content in HD group showed no significant difference compared to the ND group at 21, 28, or 42 days of age. However, at 35 days, serum CORT content in the HD group was significantly elevated ($p < 0.05$) compared with the ND group; supplementing the basal diet with AEE significantly reduced serum CORT content in the HD broilers.

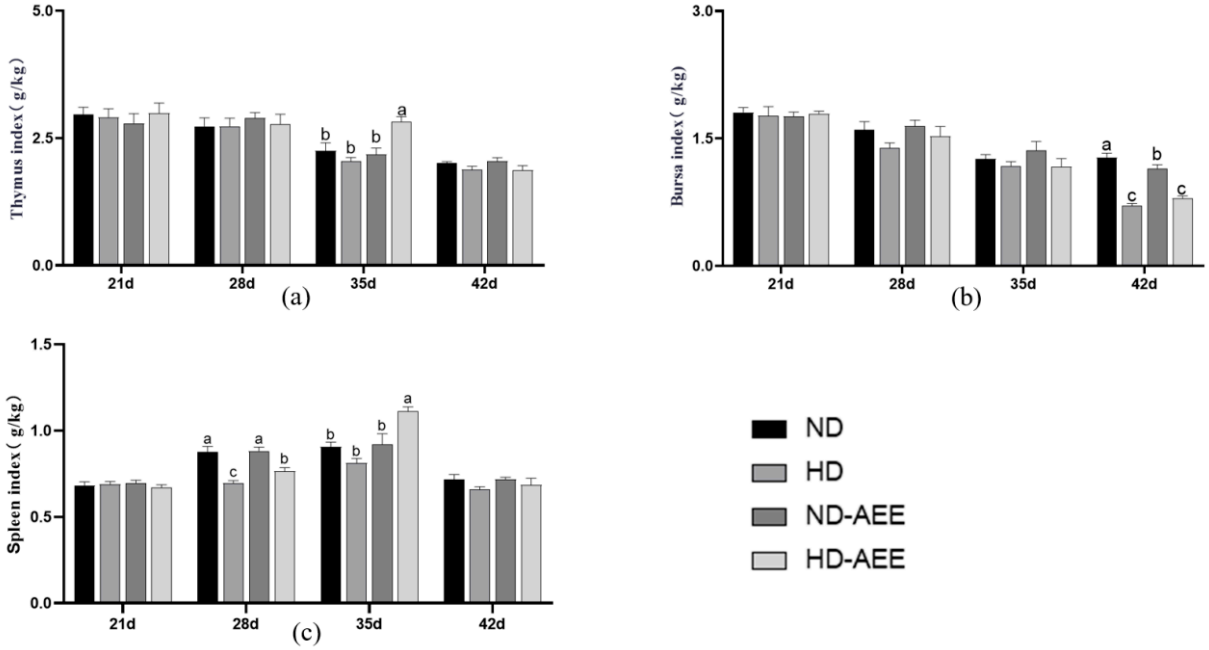

Figure 2. Effect of AEE on immune organ indices in HD broilers. (**a**) Thymus index; (**b**) Bursa index; (**c**) Spleen index. Each vertical bar represents the mean ± SEM (n = 6). Values with different letters are significantly different ($p < 0.05$).

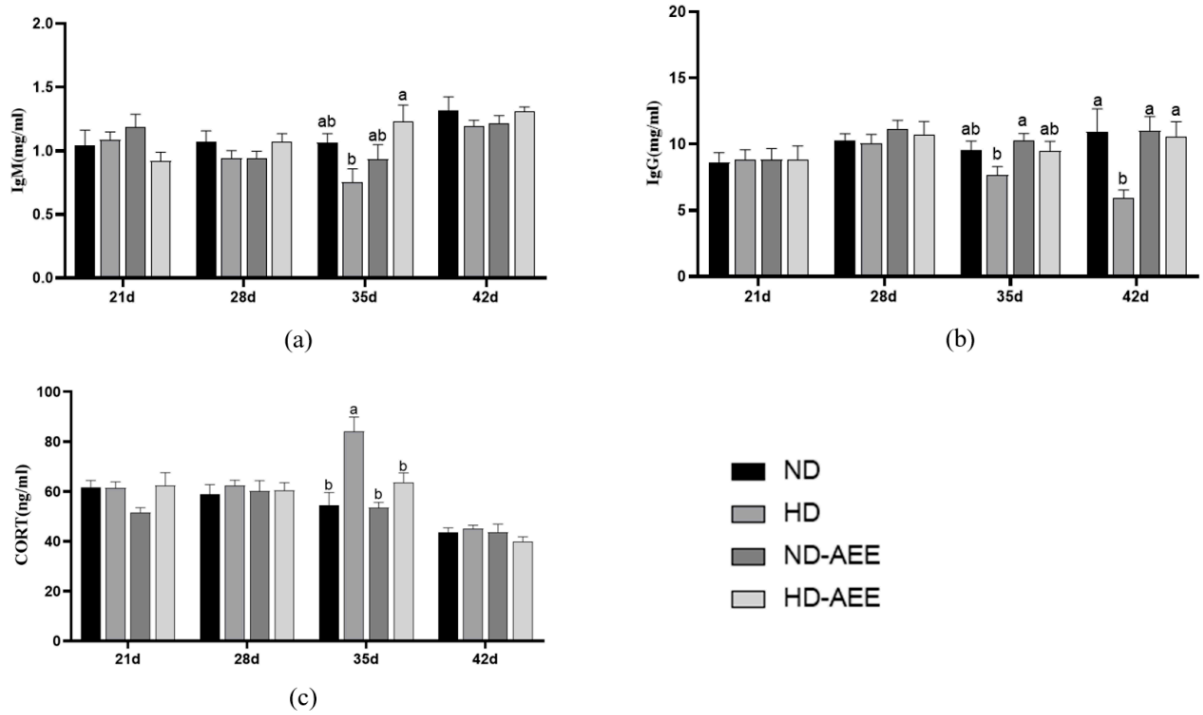

Figure 3. Effect of AEE on serum immunological indices in HD broilers. (**a**) Immunoglobulin M (IgM); (**b**) Immunoglobulin G (IgG); (**c**) Corticosterone (CORT). Each vertical bar represents the mean ± SEM (n = 6). Values with different letters are significantly different ($p < 0.05$).

3.4. Relative mRNA Expression of Inflammatory Factors in the Spleen

The effect of AEE on the relative mRNA expression of inflammatory factors in the spleen is shown in Figure 4. At 21 days, the relative expression of spleen IL-6, TNF-α, IL-1β, and IL-10 mRNA in broilers from the HD group showed no significant difference compared to the ND group. At 28 days, the relative expression of spleen IL-1β mRNA was significantly elevated in the HD group ($p < 0.05$); this increase was significantly reversed

upon the addition of AEE ($p < 0.05$). At 35 days, broilers in the HD group exhibited significantly higher relative expression of spleen IL-6, TNF-α, and IL-1β mRNA, as well as significantly lower IL-10 mRNA expression ($p < 0.05$) compared to the ND group. Supplementing AEE to the basal diet significantly reduced the relative expression of spleen IL-6 mRNA in HD broilers ($p < 0.05$). At 42 days, the relative expression of spleen TNF-α mRNA was significantly increased in the HD group ($p < 0.05$). Supplementing AEE to the basal diet significantly reduced the relative expression of spleen IL-1β mRNA expression in HD broilers ($p < 0.05$).

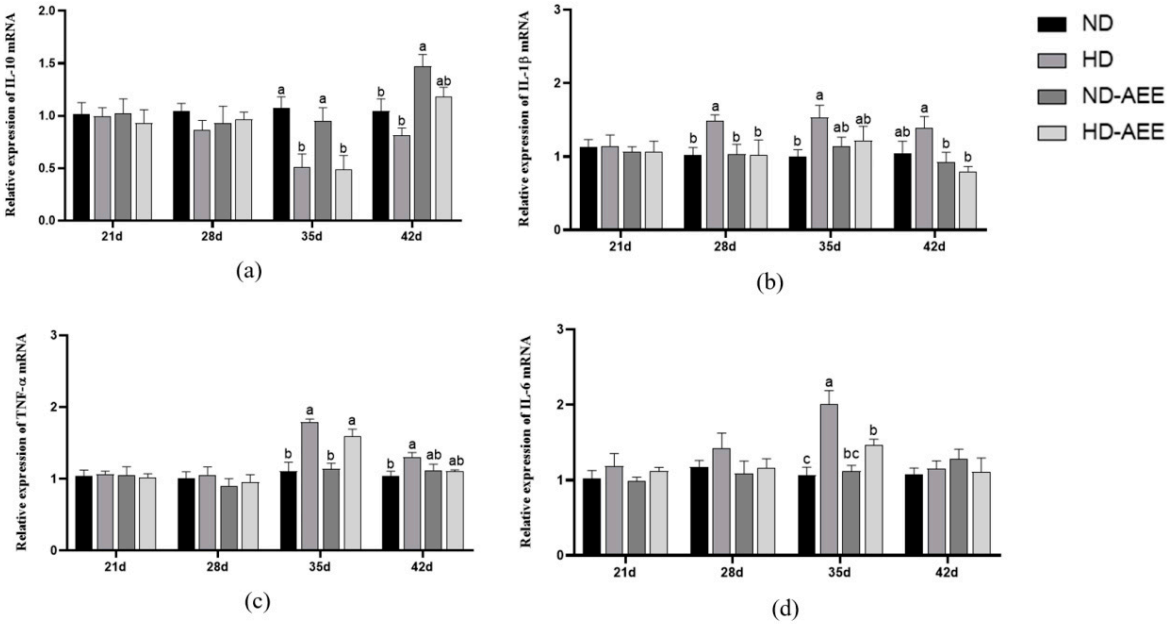

Figure 4. Effect of AEE on the expression of inflammatory factors in the spleen of HD broilers. Relative mRNA expression of (**a**) IL-10; (**b**) IL-1β; (**c**) TNF-α; (**d**) IL-6 mRNA. Each vertical bar represents the mean ± SEM ($n = 6$). Values with different letters are significantly different ($p < 0.05$).

3.5. Analysis of Differentially Expressed Genes

Analysis of differentially expressed genes (DEGs) is shown in Figure 5. At 35 days, comparison of ND vs. HD groups revealed 134 DEGs, of which 71 were up-regulated and 63 were down-regulated in the HD group. Comparison of HD vs. HD-AEE groups identified 183 DEGs, of which 67 were up-regulated and 116 were down-regulated in the HD-AEE group. Furthermore, comparison of ND vs. ND-AEE groups yielded 70 DEGs, of which 45 were up-regulated and 25 were down-regulated genes in the ND-AEE group. Venn plot analysis identified 63 shared genes between the ND vs. HD and HD vs. HD-AEE comparisons. Functional annotation of DGEs in ND-AEE relative to ND groups: On day 35, the 70 DEGs displayed significant enrichment in pathways including "starch and sucrose metabolism" and "fructose and mannose metabolism" ($p < 0.05$), with no enrichment identified for pathways pertaining to growth, immunity, or inflammation. On day 42, the 77 DEGs exhibited significant enrichment in "glycolysis/gluconeogenesis" and "amino sugar and nucleotide sugar metabolism" ($p < 0.05$), revealing no overlap with the core pathways under investigation in this study.

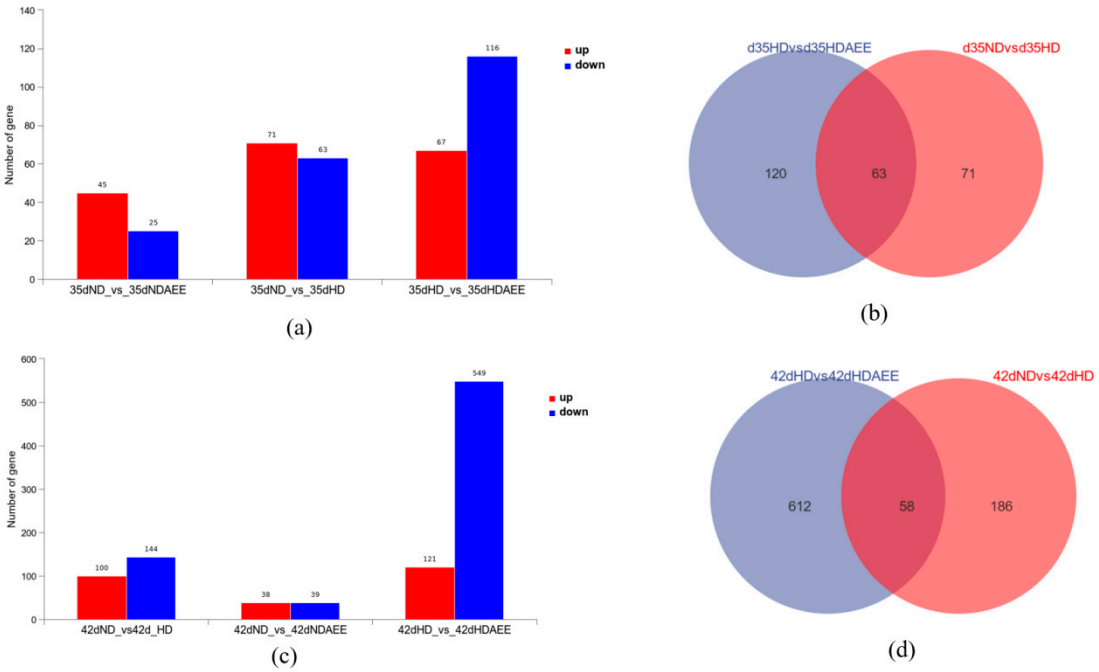

Figure 5. Effect of AEE on hypothalamic gene expression in HD broilers. (**a**) Statistics of differentially expressed genes (DEGs) at 35 days of age; (**b**) Venn diagram of DEGs at 35 days of age; (**c**) Statistics of DEGs at 42 days of age; (**d**) Venn diagram of DEGs at 42 days of age.

At 42 days, comparison of ND vs. HD groups revealed 244 DEGs, of which 100 were up-regulated and 144 were down-regulated in the HD group. Comparison of ND vs. ND-AEE groups revealed 77 DEGs, of which 38 were up-regulated and 39 were down-regulated in ND-AEE group. Comparison of HD vs. HD-AEE groups revealed 670 DEGs, of which 121 were up-regulated and 549 were down-regulated in HD-AEE group. Venn plot analysis identified 58 shared genes between the ND vs. HD and HD vs. HD-AEE comparisons.

3.6. KEGG Enrichment Analysis

At 35 days, KEGG enrichment analysis of differentially expressed genes revealed 31 significantly enriched signaling pathways in the HD group compared to the ND group, and 36 significantly enriched signaling pathways in the HD-AEE group relative to the HD group (Figure 6). At 42 days, 48 signaling pathways were significantly enriched in the HD group compared to the ND group, and 96 pathways were significantly enriched in the HD-AEE group compared to the HD group (Figure 7).

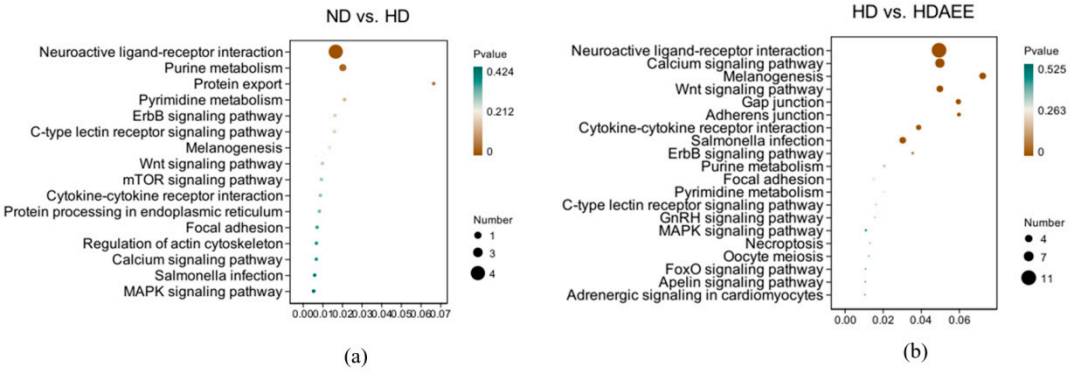

Figure 6. KEGG enrichment analysis of DEGs in the hypothalamus of 35-day-old broilers. KEGG enrichment histograms of (**a**) ND vs. HD groups; (**b**) HD vs. HD-AEE groups.

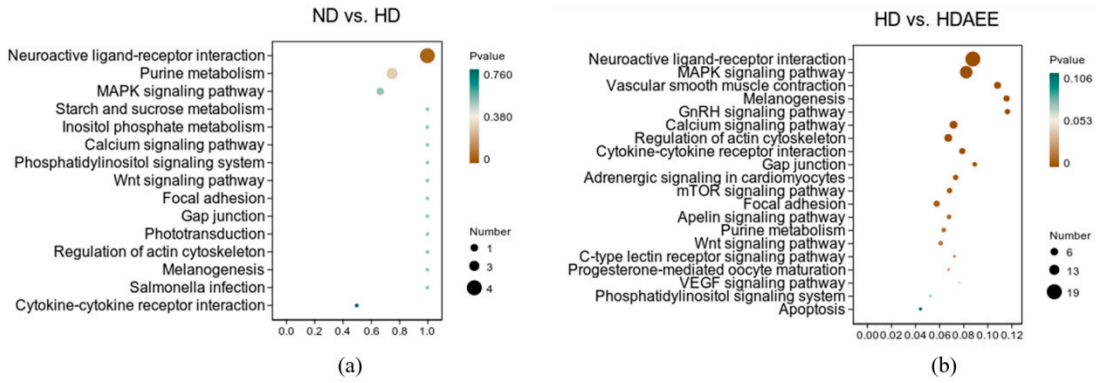

Figure 7. KEGG enrichment analysis of DEGs in the hypothalamus of 42-day-old broilers. KEGG enrichment histograms of (**a**) ND vs. HD groups; (**b**) HD vs. HD-AEE groups.

Of note, both the 35-day and 42-day comparisons (ND vs. HD and HD vs. HD-AEE) shared alterations across several key pathways. These commonly altered pathways included the MAPK signaling pathway, Wnt signaling pathway, cytokine-cytokine receptor interaction, Salmonella infection pathway, and calcium signaling pathway.

3.7. Hypothalamus-Related Gene Expression

At 35 days, the relative expression levels of PAK1 were significantly up- regulated, whereas NPY was significantly down-regulated ($p < 0.05$) in the hypothalamus of the HD group compared to the ND group (Figure 8). The relative expression levels of p38MAPK, COX-2, mPGES-1, IL-1β, TNF-α, and GHRH did not significantly differ among the groups ($p > 0.05$).

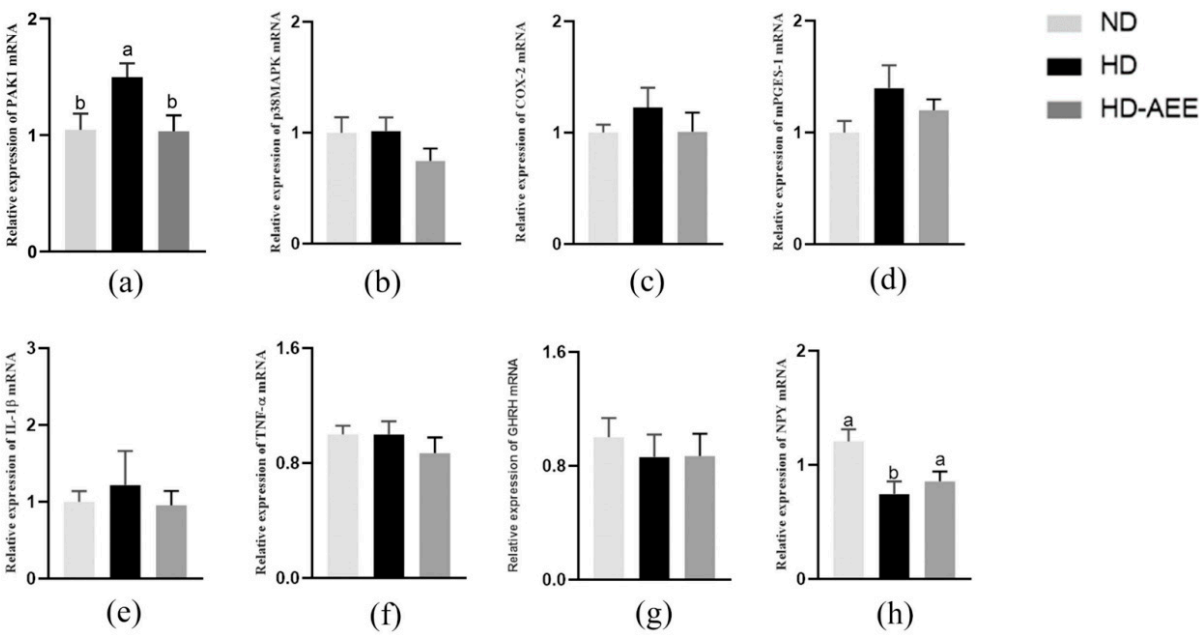

Figure 8. qRT-PCR validation of candidate genes at 35 days of age. Relative mRNA expression of (**a**) PAK1; (**b**) p38MAPK; (**c**) COX-2; (**d**) mPGES-1; (**e**) IL-1β; (**f**) TNF-α; (**g**) GHRH; (**h**) NPY. Each vertical bar represents the mean ± SEM ($n = 6$). Values with different letters are significantly different ($p < 0.05$).

At 42 days, the relative expression levels of *PAK1*, *p38MAPK*, *COX-2*, *mPGES-1*, and *IL-1β* were significantly up-regulated in the hypothalamus of the HD group compared to the ND group ($p < 0.05$) (Figure 9). Addition of AEE to the basal diet significantly reduced the expression levels of *PAK1*, *p38MAPK*, *COX-2*, *mPGES-1*, and *IL-1β* in the hypothalamus

of HD broilers ($p < 0.05$). Interestingly, addition of AEE to the basal diet significantly increased the expression level of *GHRH* compared to the ND and HD groups ($p < 0.05$).

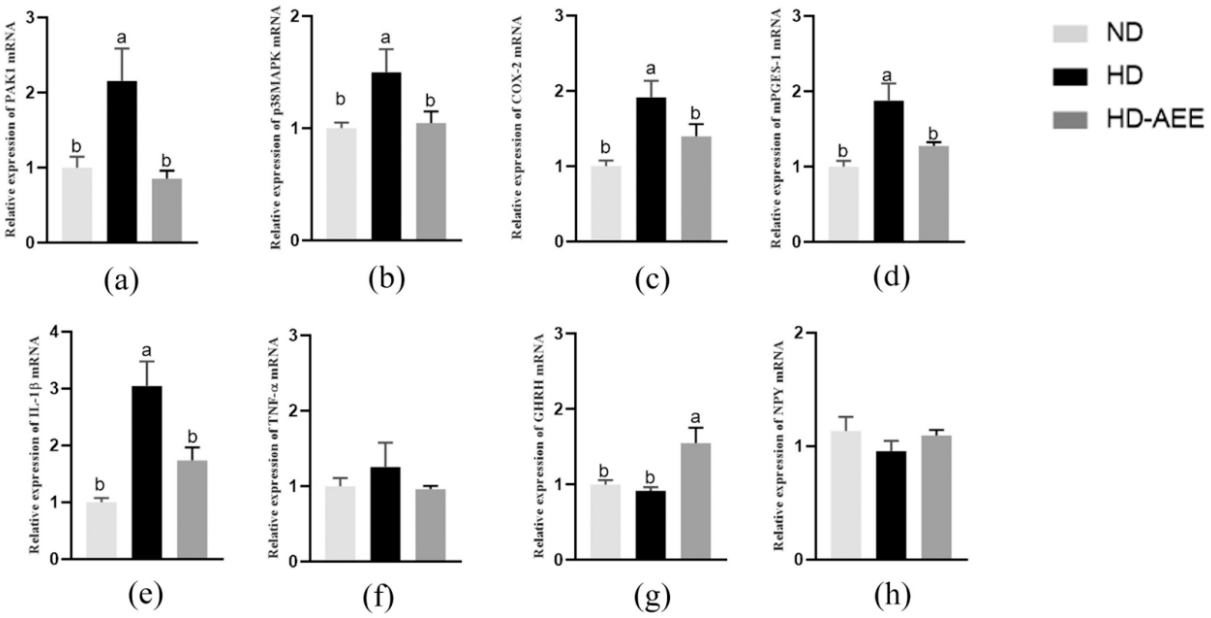

Figure 9. qRT-PCR validation of candidate genes at 42 days of age. Relative mRNA expression of (**a**) PAK1; (**b**) p38MAPK; (**c**) COX-2; (**d**) mPGES-1; (**e**) IL-1β; (**f**) TNF-α; (**g**) GHRH; (**h**) NPY. Each vertical bar represents the mean ± SEM ($n = 6$). Values with different letters are significantly different ($p < 0.05$).

4. Discussion

This study provides mechanistic insights into how environmental stress, neuroinflammation, and growth-regulatory pathways interact when broilers are reared at high stocking density and fed aspirin-eugenol ester (AEE). Broiler production is central to modern animal husbandry, and flock productivity directly determines economic returns. Although stocking density is a key determinant of broiler performance, the optimal density remains controversial. While higher density increases birds per unit area and short-term revenue, its negative impact on health and performance is well documented, including a significant reduction in both average body weight and weight gain in response to increased stocking density [22]. This phenomenon primarily originates from the high-density (HD) environment significantly constraining movement, which intensifies competition for feed and water resources, consequently impairing feed intake and nutrient assimilation efficiency. Furthermore, elevated concentrations of noxious gases, particularly ammonia, within HD environments may detrimentally affect broilers' respiratory and digestive systems, further impeding growth performance [23]. Research indicates that at densities reaching 20.3 birds/m^2, broilers exhibit significantly diminished feed intake and body weight gain, along with significantly elevated feed conversion ratios and mortality rates [8]. The present findings substantiate prior evidence: throughout the 0–42 day interval, broilers housed at elevated stocking density (HD; 22 birds/m^2) exhibited markedly diminished feed consumption and body mass accumulation, increased feed conversion efficiency, and attenuated productive outputs relative to the normal density (ND) cohort. Broilers in HD settings encountered amplified environmental pressures, wherein density-related stress functioned as a fundamental factor precipitating the documented production performance decrement.

In this experimental series, the integration of AEE into the basal diet significantly improved broiler productivity indicators, aligning with previous laboratory data [21]. First, AEE exerted a significant positive influence on body weight gain and feed conversion ratio

(FCR) throughout the entire experimental period. Furthermore, AEE exhibited greater efficacy on production performance during the 21–42 day stage relative to the 0–21 day stage. This differential effect may be attributable to broilers occupying progressively reduced space later in the growth cycle while confronting a more complex environment, thereby rendering them more vulnerable to physiological disturbances. Supplementation of the basal diet with AEE enhanced the capacity of HD broilers to withstand these adverse conditions, indicating that AEE effectively mitigates the decline in production performance induced by the HD environment. Upon exposure to stress, the hypothalamic–pituitary–adrenal (HPA) axis in broilers is activated, inducing the anterior pituitary to secrete adrenocorticotropic hormone (ACTH). ACTH subsequently stimulates adrenocortical cells to synthesize and release corticosterone (CORT) [24,25]. CORT, widely recognized as a primary physiological stress indicator, exhibited a significant up-regulation among broilers administered high doses of exogenous ACTH, thereby confirming heightened stress in an experimental model [26]. To evaluate the impact of HD environmental stress, serum CORT levels were measured. The results revealed that HD significantly elevated serum CORT concentrations in 35-day-old broilers, consistent with prior research [27]. However, HD exhibited no significant effect on serum CORT in 42-day-old broilers, likely indicating progressive adaptation to this chronic stressor. Notably, dietary supplementation with AEE in HD-exposed broilers effectively reduced serum CORT levels at 35 days, suggesting that AEE assists broilers in mitigating external environmental stress.

The spleen, thymus, and bursa of Fabricius constitute the primary immune organs in poultry, and their developmental status critically reflects overall immune function [28,29]. Elevated circulating corticosterone concentrations induce degenerative alterations in lymphoid organs, including the spleen and bursa, thereby modulating immune responses via lymphocyte depletion [30–32]. Previous studies have demonstrated a negative correlation between stocking density and spleen and bursa indices [8]. Consistent with these findings, our results indicate that high stocking density significantly reduced spleen and bursa indices, whereas the thymus index remained unaffected. However, contradictory evidence has also been reported, with some research indicating no significant impact of stocking density on broiler immune organ indices [33]. These discrepancies likely arise from variations in poultry breeds, rearing densities, experimental duration, or bird age. Notably, dietary supplementation with AEE significantly increased spleen and thymus indices in HD broilers, indicating that AEE promotes the growth and development of these critical immune organs.

Immunoglobulin content, mainly comprising IgG, IgM, and IgA, constitutes a critical indicator for assessing poultry immune function [34]. IgG primarily combats pathogenic bacteria and viruses encountered in the external environment, while inhibiting their proliferation [34]. In contrast, IgM identifies and eliminates exogenous substances during infection, thereby protecting the host from pathogen invasion. When rearing density exceeds optimal levels, broilers activate compensatory immune responses to counteract stressors such as crowding and heightened pathogen exposure [30]. Our experimental findings demonstrate that high-density rearing significantly reduced serum IgG levels in 42-day-old broilers. Furthermore, supplementation with AEE significantly increased serum concentrations of both IgG and IgM in these HD broilers. This result indicates that AEE effectively enhances immune function in densely stocked broilers. TNF-α and IL-1β play pivotal roles in mediating local and systemic inflammatory responses, subsequently activating cytokines such as IL-6 and IL-10. IL-6 acts as a key mediator in chronic inflammation [35,36], whereas IL-10 exerts potent inhibitory effects, suppressing inflammatory cascades and maintaining immune homeostasis [37]. Research on the relationship between rearing density and splenic inflammatory factor expression remains limited. Our study demonstrates that

high-density feeding significantly elevates the relative expression of pro-inflammatory factors IL-6, TNF-α, and IL-1β in the spleen while suppressing the anti-inflammatory factor IL-10, thereby inducing inflammatory responses. Based on these immune indicators, we hypothesized that the detrimental effects of late-stage high-density farming on broilers may primarily stem from systemic inflammatory responses. Hypothalamic transcriptome analyses at days 35 and 42 revealed significant KEGG pathway enrichment for both the ND vs. HD and HD vs. HD-AEE comparison groups. These analyses consistently implicated the MAPK signaling pathway, the Wnt signaling pathway, and cytokine-cytokine receptor interactions pathways, all of which are intrinsically associated with inflammatory cascades.

The mitogen-activated protein kinase (MAPK) pathway constitutes a highly conserved and functionally critical signaling module in eukaryotic organisms. As an essential intracellular signaling cascade, it plays a fundamental role in regulating diverse biological processes, including cell growth, differentiation, inflammation, and stress responses. Numerous studies have demonstrated the involvement of the MAPK pathway in leptin signaling [38], lipocalin receptor signaling [39], growth hormone signaling [40], and inflammatory regulation [41]. Notably, the p38MAPK pathway, a principal branch of the MAPK cascade, responds robustly to stress stimuli, including cytokines and environmental stressors, and is closely associated with cellular survival and inflammatory responses. Concurrently, the Wnt signaling pathway exerts substantial regulatory control over cell survival, proliferation, and differentiation processes [42]. Furthermore, Wnt signaling is indispensable for neuroendocrine regulation within the hypothalamus, significantly influencing body weight and food intake [43]. Hypothalamic inflammatory responses can be directly mediated by activated microglia [44]. In fact, the accumulation of inflammatory stimuli within the hypothalamus activates these microglia, thereby initiating the inflammatory cascade. Notably, in this study, transcriptome analysis revealed significant alterations in PAK1 expression within the MAPK pathway in broilers at both 35 and 42 days. Evidence indicates that PAK1 interacts with and activates both MAPK and NF-κB signaling pathways, potentially acting as an upstream regulatory component for both. Through these pathways, PAK1 likely modulates downstream gene expression. Substantial literature links both MAPK and NF-κB signaling pathways to the regulation of neuroinflammation; specifically, pharmacological inhibition of these pathways reduces the expression of inflammatory factors (such as COX-2 and iNOS) in activated microglia [45–47]. PAK1 has been demonstrated to activate the p38MAPK pathway, which is implicated in apoptosis and tumor angiogenesis [48]. Importantly, PAK1 can also regulate COX-2 expression via the p38MAPK pathway, thereby contributing to the control of neuroinflammation. Previous studies [49] have documented that AEE alleviates inflammatory responses in broilers, such as mitigating intestinal inflammatory injury.

A significant limitation of this study was that only mRNA expression levels were measured, with no assessments of protein abundance, post-translational modifications (e.g., p38MAPK phosphorylation), or enzymatic activity (e.g., COX-2 catalytic function). Since mRNA expression does not reliably reflect protein function, the observed down-regulation of PAK1/p38MAPK/COX-2 pathway-related genes cannot be equated to reduced protein levels or functional activity. Furthermore, no functional manipulations (PAK1 over-expression/knockdown, COX-2-specific inhibition) were conducted to confirm a causal association between pathway down-regulation and the beneficial effects of AEE, with only an associative relationship being established. Furthermore, under normal stocking density (ND, 14 birds/m^2), RNA sequencing analysis identified DEGs in the ND-AEE group. However, these DEGs were predominantly enriched in fundamental metabolic processes rather than pathways associated with growth, immunity, or inflammation. Correspondingly, no substantial phenotypic disparities (including growth

performance, immune organ indices, serum immunoglobulins, or splenic inflammatory factors) were detected between the ND and ND-AEE groups. This finding suggests that the beneficial effects of AEE are stress-dependent and possess limited practical utility for broilers reared under optimal, non-stressed conditions. Future studies should validate the findings via Western blot (protein quantification), functional assays (COX-2 activity, PGE2 production), genetic/pharmacological interventions (pathway modulation), and exploration of PAK1 upstream regulators to confirm AEE's direct regulatory role in the PAK1/p38MAPK/COX-2 axis.

The present study demonstrated that dietary supplementation with AEE was associated with significantly reduced mRNA expression of PAK1 in the hypothalamus of 35-day-old broilers reared under high-density conditions. Furthermore, AEE was correlated with decreased mRNA expression of *PAK1*, *p38MAPK*, *COX-2*, *mPGES-1*, and *IL-1β*, as well as increased mRNA expression of *GHRH*, in the hypothalamus of 42-day-old HD broilers. These gene expression changes correlate with AEE-induced improvements in growth and immune function, suggesting a potential link to the modulation of hypothalamic inflammation—but do not confirm direct pathway inhibition or functional regulation.

5. Conclusions

This research explored the impacts of 0.01% AEE on broiler chickens reared under high stocking density conditions. The findings demonstrated that high—density rearing triggered hypothalamic neuroinflammation in broilers, concurrently suppressing their growth performance and compromising immune function. Dietary supplementation with AEE effectively mitigated the high—density—induced inflammatory response by down-regulating the mRNA expression of hypothalamic PAK1 and genes related to its downstream p38MAPK/COX-2 signaling axis (including PAK1, p38MAPK, COX-2, mPGES-1, and IL-1β), and simultaneously up-regulating the expression of the growth—associated gene growth hormone—releasing hormone. This regulatory effect of AEE enhanced broiler weight gain, feed utilization efficiency, and immune organ development, and also elevated serum immunoglobulin levels. The beneficial effects were more pronounced during the late growth stage (21–42 days of age). However, AEE had no significant impact on broilers reared under normal density, and its underlying regulatory mechanism still necessitates further validation at the protein and functional levels.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture16010038/s1, Supplementary Table S1: Growth performance parameters of broilers in different groups (mean ± SEM, $n = 6$); Supplementary Table S2: Growth performance parameters of broilers in different groups (mean ± SEM, $n = 6$); Supplementary Table S3: Immune organ indices of broilers in different groups (mean ± SEM, $n = 6$, mg/g); Supplementary Table S4: Serum immunoglobulin and corticosterone (CORT) concentrations of broilers in different groups (mean ± SEM, $n = 6$).

Author Contributions: Conceptualization: D.B., Y.Z. (Yi Zhang) and Y.M.; Data curation: X.Z., Y.W., B.Z. and X.X.; Formal analysis: X.Z., Y.W., B.Z. (Bo Zheng) and X.X.; Funding acquisition: Y.M.; Investigation: D.B., Y.Z., X.Z., Y.W., B.Z. (Bo Zheng) and X.X.; Methodology: W.Z. and F.G.; Project administration: W.Z. and F.G.; Resources: Y.Z. (Yushu Zhang). and B.Z. (Bingkun Zhang).; Software: X.Z., Y.W., B.Z. (Bo Zheng) and X.X.; Supervision: Y.M.; Validation: B.Z. (Bo Zheng) and X.X.; Visualization: X.Z. and Y.W.; Writing—original draft: D.B. and Y.Z. (Yi Zhang); Writing—review and editing: Y.Z., (Yi Zhang) W.Z., F.G., Y.Z. (Yushu Zhang), B.Z. (Bingkun Zhang) and Y.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Animal Care and Use Committee of Henan University of Science and Technology. The management and experimental procedures of the experiment animals complied with the regulations of the Institutional Animal Care and Use Committee.

Informed Consent Statement: Not applicable.

Data Availability Statement: The corresponding author will provide access to the data sets generated or analyzed during this study upon reasonable request.

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Conflicts of Interest: The authors declare that they have no competing interests.

Abbreviations

AEE	Aspirin Eugenol Ester
HD	High Stocking Density
ND	Normal Stocking Density
BW	Body Weight
ADG	Average Daily Gain
FI	Feed Intake
FCR	Feed Conversion Ratio
COX-2	Cyclooxygenase-2
mPGES-1	Prostaglandin E Synthase 1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
TNF-α	Tumor Necrosis Factor-α
NPY	Neuropeptide Y
CORT	Corticosterone
DEGs	Differentially Expressed Genes
KEGG	Kyoto Encyclopedia of Genes and Genomes
HPA	Hypothalamic–Pituitary–Adrenal
NF-κB	Nuclear Factor-Kappa B
ANOVA	Analysis of Variance
PGE2	Prostaglandin E2

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