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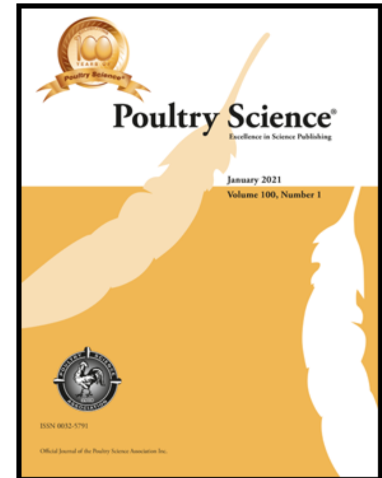

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Running title: Ethoxyquin attenuates heat stress-challenged broilers

Ethoxyquin attenuates enteric oxidative stress and inflammation by promoting cytokine expressions and symbiotic microbiota in heat-stressed broilers

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ABSTRACT

Intestinal oxidative stress in broilers is produced by chronic heat stress (HS) and has a negative impact on poultry performance as it induces intestinal inflammation and promotes the invasion of gram-negative bacteria, such as bacterial lipopolysaccharide (LPS). Therefore, dietary inclusion of the antioxidant compound, ethoxyquin (EQ), could improve enteric antioxidant capacity, immune responses, and the epithelial barrier, and maintain the symbiotic gut microbiota community. To investigate the effects of EQ supplementation on alleviating enteric oxidative stress in heat-stressed broilers, 200 one-day-old male Ross 308 broilers were randomly assigned to four groups (n = 50 chicks/group; n = 10 chicks/replicate) and fed a basal diet supplemented with 0 (CT), 50 (EQ-50), 100 (EQ-100), and 200 (EQ-200) mg EQ/ kg^{-1} for 5 weeks. The chicks were raised in floor pens inside the broiler farm at a temperature and humidity index (THI) of 29 from day 21 to day 35. Growth performance traits, relative organ index, hepatic antioxidant enzymes, serum immunity, total adenylate, and cytokine activities were improved in the EQ-50 group (linear or quadratic $P < 0.05$), promoting the relative mRNA expression of cytokine gene-related anti-inflammatory and growth factors. A distinct microbial community colonised the gut microbiota in the EQ-50 group, with a high relative abundance of *Lactobacillus*, *Ligilactobacillus*, *Limosilactobacillus*, *Pediococcus*, *Blautia*, and *Faecalibacterium* compared to the other groups. Dietary supplementation with 50 mg EQ/ kg-1 for 5 wk attenuates enteric oxidative stress and intestinal inflammation by enhancing serum immune and cytokine content (IgG, IL-6, and TGF-β,) and symbiotic microbiota in heat-stressed broilers. EQ promotes the expression of *Hsp70*, *SOD2*, *GPx 4*, *IL-6,* and *IGF-1* cytokine gene-related anti-inflammatory and growth factors in heat-stressed hepatic broilers. Collectively, EQ-50 could be a suitable feed supplement for attenuating enteric oxidative stress and intestinal inflammation, thereby promoting the productivity of heat-stressed broilers.

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Keywords: Ethoxyquin, heat-stressed broilers, enteric oxidative stress, antioxidant capacity, symbiotic gut microbiota.

INTRODUCTION

Oxidative stress (OS) in the poultry gastrointestinal tract is caused by nutritional, pathological, and environmental factors, such as heat stress (HS), and has a negative impact on poultry performance due to the excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Mishra and Jha, 2019). Both ROS and RNS can contribute to lipid peroxidation (LP), especially cell membrane lipids and lipoproteins, as they are rich in polyunsaturated fatty acids (PUFA), which mediate the induction of intestinal inflammation (Sahoo, et al., 2023). In addition, OS associated with environmental HS in cells and tissues results from an imbalance between free radical production and endogenous antioxidant defences, leading to LP, protein nitration, DNA damage, and apoptosis (Mishra and Jha, 2019). The first line of defence against OS is the intestinal epithelium because it contains an extensive antioxidant defence system, including enzymes (CAT, SOD, GSH, and GPX) and non-enzymatic scavengers such as transient ions (e.g. Fe2+ and Cu2+) or flavonoids (Tang, et al., 2019). However, chronic HS causes intestinal inflammation by invading gram-negative bacteria, such as bacterial lipopolysaccharide (LPS), also known as endotoxins, that enter the bloodstream and induce intestinal inflammation, necrosis, and ischaemia (Bertani and Ruiz, 2018). Heat-stressed broilers show reduced intestinal structure (crypt depth, mucosal area, and villus height), epithelial cell injury, and hyperpermeability, leading to bacterial LPS efflux from the intestinal lumen into the circulatory system and affecting the organ systems (Ayo and Ogbuagu, 2021).

Redox signalling in the host regulates symbiosis between the gut microbiota and the intestine (Neish and Jones, 2014). The intestinal antioxidant capacity identifies the pattern of motility,

proliferation, and lifestyle of the gut microbial community (bacteria, archaea, fungi, and ciliated protozoa) as well as the modification of mucosal cell surfaces (Chen, et al., 2021; Elokil, et al., 2020a). Through a symbiotic relationship with the host, bacteria can produce essential amino acids and vitamins and regulate digestion to shape host health and function. In poultry research, the pattern diversity of gut microbiome communities has been used as a novel molecular marker to explore the microbiome associated with candidate performance, including weight gain (Elokil, et al., 2022), egg production (Elokil, et al., 2020a), immunity (Elokil, et al., 2020c), and fertility (Elokil, et al., 2020b).

Dietary inclusion of antioxidant compounds in heat-stressed broilers reduces intestinal free radicals, enhancing intestinal mucosal integrity and maintaining a symbiotic gut microbiota community. Ethoxyquin (EQ; 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) is widely used in animal feed to protect against lipid peroxidation (LP) (Błaszczyk, et al., 2013). Considering that the dietary levels of EQ tolerated by growing chickens and breeders reported in the literature are 500–750 mg EQ/kg feed, the suggested concentration of 50-200 mg EQ/kg could be considered safe for chickens and breeders (Błaszczyk et al., 2013; Tadokoro, et al., 2022). The European Commission has confirmed that EQ is a safe and potent antioxidant that is not genotoxic to all animal species when used at 50 mg EQ/kg feed which can be considered safe for chickens (Additives and Feed, 2015). Recently, Monsanto Company (USA) performed a series of tests on EQ which demonstrated its safety, and it was approved by the United States Food and Drug Administration (FDA) for use in animal feed, including all animal species. In addition, EQ may help mitigate the detrimental effects of OS associated with environmental HS in animals and maintain bird growth, performance, and survival by protecting gut integrity, limiting the translocation of bacterial pathogens, and modulating the production of pro-inflammatory

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cytokines (de Koning, 2002; Tian, et al., 2021). The previous studies have shown the beneficial effects of EQ as an antioxidant used in animal feed in different species, such as broiler (Kuttappan, et al., 2021), human (Tadokoro, et al., 2022), pigs (Gai, et al., 2018), fish (Wang, et al., 2015), rat (Iskusnykh, et al., 2021), cow (Wang, et al., 2018), and calves (Wei, et al., 2023). It has also been used as feed for aquatic animals, including Atlantic salmon (Bernhard, et al., 2019), zebrafish (Pradhan, et al., 2020), and red carp (Li, et al., 2019). Therefore, the aim of the present study was to elucidate the effect of 5 weeks of EQ supplementation on alleviating oxidative stress and intestinal inflammation by promoting cytokine expression and symbiotic microbiota in heat-stressed broilers.

MATERIALS AND METHODS

Location and Ethics Statement

The study was conducted at chicken farm of the Animal Production Department, Faculty of Agriculture at Benha University. Samples analysis was performed in the laboratory of National Organization for Drug Control, and Laboratory of Molecular Biology, National Research Centre, Egypt, and in collaboration with the College of Animal Science and Technology, Huazhong Agricultural University, Wuhan, Hubei, PR. China. All experimental procedures were reviewed and approved (BUFAG-APD230710) by Animal Production Department, Faculty of Agriculture, Benha University, Egypt. Animals were handled in accordance with the guidelines described by the Animal Care Committee of Qalyubiya Province, Egypt. In this study, all efforts were made to minimize the animal suffering and was carried out in compliance with the ARRIVE guidelines.

Animals, experimental design and diets

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A total of 200 one-d-old of Ross 308 male broiler chicks were randomly allocated into 4 dietary treatments (n = 50 chicks/group) each consisting of 5 replicates (n = 10 chicks/ replicate), as follows: CT group, without EQ supplementation as control group; EQ-50 group, supplemented with 50 mg EQ / kg^{-1} diet; EQ-100 group, supplemented with 100 mg EQ / kg^{-1} diet; EQ-200 group, supplemented with 200 mg EQ / kg^{-1} diet for 5 wk of experimental period. All EQs levels were mixed into the diet pellets. Chicks were raised in one room containing 20 floor pens (10 chicks/pen) with equal dimensions (150 cm length × 120 cm width × 60 cm height) and the pen floor was covered with a 5 cm deep bedding of sawdust. The value of temperature-humidity index (THI) inside the chicken house was assigned at 29 THI, which is higher than the maximum value of the livestock safety index (optimum THI value of 23). Briefly, the initial temperature was fixed at 32°C on d 1, and gradually lowered until it reached 23°C on d 21. After that, the temperature was increased gradually until reaching 32 ± 2°C for 14 d, and relative humidity was extended from 65% to 75%. On d 7 and d 21, live Newcastle disease vaccine was administered to the birds. The purity of EQ was more than 90%, purchased from Henan Zhengtong Food Technology Co., Ltd (Henan, China).

Chicks were allowed *ad libitum* access to experimental diets of starter crumbles (0-14 days), grower pellets (15-28 days), and finisher pellets (28-35 days). The experimental diets were formulated to meet nutrient requirements for Ross 308 broiler nutrition specifications (Aviagen, 2014). The feed ingredient composition and estimated nutrient levels are presented in Table 1. To perform dietary nutrient analysis, the diets were ground using a Retsch laboratory mill (Retsch ZM 200, Germany) to 1- and 0.5-mm screen-hole sizes. Analyses, including moisture, total starch, crude protein, fat, ash, and insoluble and soluble fiber of the ground feed according to

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Zhang (2007). The analysis involved the use of an automated Kjeltec 8400 Analyzer Unit (FOSS Analytical AB, Hoganas, Sweden) and an amino acid analyzer (L8900, Hitachi, Tokyo, Japan).

Growth Performance

Chicks were weighed on days 0, 21, and 35 before feeding in the morning. Feed intake (FI) and the number of dead birds were recorded daily. The average daily feed intake (ADFI), average daily weight gain (ADG), and feed conversion rate (FCR) were calculated based on the following formulas: ADFI (g/d) = total food intake (g)/feed days (d); ADG (g/d) = (final weight - initial weight) (g)/feed days (d); FCR (g/g) = ADFI (g/d)/ADG (g/d). Survivability rates (%) = (Number of surviving birds at the end of experimental period/initial number of birds) × 100. At the end of the experimental period (age 35 days), a total of 10 birds/group (2 birds in each replicate) were selected, representing the average body weight of each group. The chicks were individually weighed and then slaughtered, and the carcass and internal organs of the liver, gizzard, proventriculus, heart, and intestine were weighed individually to calculate their relative weights to live body weight (LBW) and expressed vis-à-vis the LBW weight (organ weight/LBW) x100.

Biochemical analysis

Liver concentrations (n= 10/group) of malondialdehyde (MDA), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), glutathione disulfide (GSSG), nitric oxide (NO), adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) were measured by HPLC method (Agilent HP 1200 series HPLC apparatus, Midland, ON, Canada). All standards were obtained from Sigma-Aldrich Chemie GmbH Export Co. Ltd., Taufkirchen, Germany. Additionally, the liver activities of catalase (CAT) and superoxide dismutase (SOD) were measured by spectrophotometric method, at 420 nm, for 1 min, on a UV-Vis Shimadzu spectrophotometer. The serum (n= 10/group) contents of immunoglobulin A (IgA),

immunoglobulin G (IgG), immunoglobulin M (IgM), tumor necrosis factor-α (TNF-α), transforming growth factor-beta (TGF-β), and the interleukins IL-6 and IL-10 were measured colorimetrically with commercial kits purchased from Nanjing Jiancheng Inst. of Bioengineering, (Nanjing, China). For musculus longissimus dorsi (MLD) amino acid profile assay, Each MLD muscle sample (n = 10) was weighed and homogenized in 75% aqueous HPLC grade methanol (10% w/v). The homogenate was spun at 4000 rpm/10 min, and the supernatant was dried with a vacuum (70 Millipore), at room temperature, and used for amino acid (AA) assay by HPLC, using the precolumn PITC derivation technique.

Hepatic expression of peptide growth factor and cytokines-related genes

Total RNA (n= 5/group) was isolated from homogenised liver tissue samples using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity and quality of the RNA samples were determined using a NanoDrop spectrophotometre and gel electrophoresis. Genomic DNA was removed by DNase, and 1 μg of each RNA sample was used to generate cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). The reaction mixture (10 μL) for qPCR contained 5 μL of SYBR (Takara, Japan), 0.4 μL of forward and reverse primer, 3.2 μL of ddH_2O, and 1 μL of template cDNA on a 7500 RealTime PCR system (Applied Biosystems, USA). The qPCR conditions were as follows: pre-denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 20 s. The primer sequences are listed in Table 2, and β-actin was used as a reference for qPCR. All samples were run in triplicate with standard deviation (SD) and threshold cycle (Ct) values not exceeding 0.5. Gene expression levels were quantified using the ΔΔCt method ($R = 2^{-\Delta\Delta Ct}$) where R is the relative expression of the gene and ΔCt is the value obtained by subtracting the Ct value for *β-actin* mRNA from the Ct value of the target mRNA.

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Microbial genomic DNA extraction and 16S rRNA gene sequencing

Total bacterial genomic DNA (n=7/group) was extracted from 250 mg of each caecum digesta sample using Fast DNA stool mini kit (QIAmp DNA Stool mini Kit; QIAGEN, Hilden, Germany), following the manufacturer's instructions, and stored at -20 °C for further analysis. The quantity and quality of extracted eligible DNA fragments (OD 250/270 ranged from 1.7 to 2.0) were measured using a NanoDrop ND-1000 spectrophotometre (Thermo Fisher Scientific, Waltham, MA, USA) and 1% agarose gel electrophoresis, respectively. The PCR amplification of the hypervariable regions V3-V4 of the bacterial 16S rRNA gene was performed using the forward primer 338F (5′- CCTAYGGGRBGCASCAGGNG-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWT CTAAT-3′). Only sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR mixture contained 5 × *TransStart* FastPfu buffer 4 μL, 2.5 mM dNTPs 2 μL, forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, *TransStart* FastPfu DNA polymerase 0.4 μL, template DNA 10 ng, and fnally ddH_2O up to 20 μL. PCR conditions were set as follows: initial denaturation at 98°C for 2 min, followed by 25 cycles of denaturation at 98°C for 15 s, annealing at 55°C for 30 s, and extension at 71°C for 30 s, with a final extension of 5 min at 72°C. PCR was performed in triplicate and amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN, USA) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). The concentration of purified amplicon library of each sample was assigned above 0.5 ng/uL, and amplicons were pooled in equimolar and paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols of Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). More than 30,000 clean reads were obtained from each purified amplicon library. Each sequencing process was conducted by Personalbio Co., Ltd., and the results are

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publicly available in the Penzano Biomicrobiology Group report under opening number MBP20230904-3.

Sequencing quality assessment

Raw sequencing reads with exact matches to the barcodes were assigned to the respective samples and identified as valid sequences using fast length adjustment of short reads (FLASH). The chimeric sequences were identified with an average PHRED score of less than 25 and a sequence length shorter than 10 bp and were eliminated to obtain valid reads. Sequences that overlapped in the region between R1 and R2 were assembled based on their overlapping sequences. Operational taxonomic units (OTUs) with 97% similarity were clustered using UPARSE (version 7.1, http://drive5. com/uparse/), and the QIIME, v1.8.0 pipeline was employed to process the sequencing data. The taxonomy of each OTU representative sequence was analysed using the ribosomal RDP database (http://rdp.cme.msu. edu/g) compared with the Silva 138 database. The α-diversity was described using the Shannon index and Chao index.

Sequencing data analysis

Alpha diversity analysis, including Shannon, Simpson, Chao1, and ACE indices, as well as community uniformity, was applied to identify the drivers of variation in microbial community structure among samples. Beta diversity analysis with principal component analysis (PCA), principal coordinate analysis, NMDS, and UPGMA cluster analysis was used to obtain a comparative analysis of intergroup/group differences in the UniFrac distance. For β-diversity, ANOSIM was used to calculate PCA, PCO-A and NMDS with R value based on weighted and unweighted-UniFrac distance. To predict the association function of 16S rRNA gene sequences, PICRUSt was performed based on Tax4Fun of the Kyoto Encyclopedia of Genes and Genomes (KEGG) and then visualised using the statistical analysis of metagenomic profiles (STAMP)

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software package. According to the predicted abundance distribution of each functional group in each sample, a histogram was constructed for display. Finally, R software was used for cluster analysis of the top 50 most abundant functional predictions in each sample, as presented in the heat map.

Statistical Analysis

The effect of dietary level of EQs supplementation was analyzed by one-way ANOVA (SAS 9.1. 2004, SAS Institute, Cary, NC). Orthogonal polynomial contrasts were used to estimate the linear and quadratic effects of the increasing dietary EQs level, and probability level at 0.05 was adopted to identify significance. Replicate was used as the experimental unit (n = 10). Data for each variable are presented as means, along with the SEM for n = 10, based on the ANOVA error mean square. Duncan's multiple range test were done to compare the differences between means.

RESULTS

Effect of dietary supplementation of EQ on growth performance and relative organ index of heat-stressed broilers.

The results presented in Table 3 show that the growth performance of heat-stressed Ross 308 male broiler chicks was affected by dietary EQ levels. The results reveal that supplementing broiler diets with 50, 100, and 200 mg EQ/kg had significant linear and quadratic effects on FBW, ADG, and survival. The average FBW and ADG were affected by EQ dietary inclusion, which was significantly increased in the EQ-50 group compared to the other groups. The highest average FBW (2318.5 g) was observed in the EQ-50 group ($P < 0.05$, linear or quadratic; $P < 0.05$). In addition, ADG significantly increased ($P < 0.01$, linear or quadratic $P < 0.05$) in chicks in the EQ-50 group compared to the other groups. The average FCR showed significant response ($P < 0.05$) to the EQ substitution. Carcasse yield and relative organ index results are shown in Table 4. There

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were statistically significant differences ($P < 0.05$, linear or quadratic $P < 0.05$) in the average carcase yield and relative organ weights, including those of the liver, gizzard, and heart between the groups. The highest average (linear or quadratic, $P < 0.05$) of carcase yield (1785.05 g) and index weights for heart (0.63 %) was observed in the EQ-100 group. In addition, the highest average ($P < 0.05$) of index weights for liver (2.43 %), gizzard (2.01 %) were measured with the EQ-200, and CT groups, respectively (Table 4).

Effect of dietary supplementation of EQ on hepatic antioxidant activities and total adenylate of heat-stressed broilers.

The antioxidant activities and total adenylates (ATP, AMP, and ADP) in the liver are shown in Table 5. The results reveal significant differences among the groups in the liver concentration of MDA, which was significantly lower ($P < 0.05$, linear $P < 0.05$) in the EQ-50 group than in the other groups. A significant increase in the liver activity of NO ($P < 0.05$, linear or quadratic $P < 0.05$) and CAT ($P < 0.05$, quadratic $P < 0.05$) was observed in the EQ-50 group compared to the other groups. The highest activity of SOD ($P < 0.05$, linear or quadratic $P < 0.05$) and concentration of ATP ($P < 0.05$, linear or quadratic $P < 0.05$) in the liver tissue were measured in the EQ-100 group. In contrast, the liver concentrations of ADP ($P < 0.05$) and AMP ($P < 0.05$, quadratic $P < 0.05$) were significantly increased in the CT and EQ-200 groups, respectively. The concentration of the digestive enzymes significantly increased ($P < 0.05$) the lipase and amylase levels in the mucosal tissue of the EQ-50 group, as shown in Table 5.

Effect of dietary supplementation of EQ on serum immunity and cytokine activities of heat-stressed broilers.

Serum immunity and cytokine activity results are presented in Table 6. The results reveal significant differences in the serum concentrations of IgA ($P < 0.05$), IgG ($P < 0.05$, linear P <

0.05) and IgM ($P < 0.01$, quadratic $P < 0.05$), which increased in the EQ-100, EQ-50 and EQ-200 groups, respectively. In addition, serum concentrations of ALT and AST were significantly higher ($P < 0.05$) in the CT and EQ-200 groups than in the other groups. The highest average serum concentrations of IL-10 and TNF-α (linear $P < 0.05$) were observed in the EQ-100 group. There were increased in the serum content of IL-6 and TGF-β ($P < 0.05$, linear $P < 0.05$) in the EQ-50 group compared with the other groups (Table 6).

Effect of dietary supplementation of EQ on essential amino acid profile in MLD basis on dry matter of heat-stressed broilers.

The effects of dietary supplementation with EQs on the essential amino acid (AA) profile of MLD in dry matter of heat-stressed broilers are shown in Table 7. There was a significant increase in the concentrations of aspartic acid ($P < 0.05$, quadratic $P < 0.05$), alanine ($P < 0.05$, linear or quadratic $P < 0.05$), and threonine ($P < 0.05$, linear or quadratic $P < 0.05$) in the CT group compared to those in the other groups. Similarly, the highest MLD of leucine ($P < 0.05$, linear $P < 0.05$) and glutamic acid ($P < 0.05$) were observed in the EQ-50 group. The highest concentrations of valine ($P < 0.05$), methionine ($P < 0.05$), and arginine ($P < 0.01$, linear $P < 0.01$, and quadratic $P < 0.05$) were observed in the EQ-100 group. In addition, the MLD concentrations of lysine, isoleucine, and glycine were significantly higher ($P < 0.05$) in the EQ-200 group than those in the other groups. However, there were no significant differences in the MLD concentrations of histidine and tyrosine between groups (Table 7).

Effect of dietary supplementation of EQ on cytokines gene-related anti-inflammatory and growth factors of heat-stressed broilers.

The relative mRNA expression levels of cytokine gene-related anti-inflammatory and growth factors are shown in Table 8. The expression of *GPx 4* ($P < 0.01$, linear $P < 0.05$), *IGF-1* (P <

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0.01), and *IL-6* ($P < 0.01$) was significantly upregulated in the liver tissue of the EQ-50 group compared to the other groups. The expression of *GH* ($P < 0.05$) was significantly upregulated in the liver tissue of the EQ-100 group compared with that in the other groups. In addition, the expression of *Hsp70* was significantly ($P < 0.05$) upregulated in the liver tissue of the EQ-200 group compared to that in the other groups. In contrast, the expression of *Il-10* genes was significantly downregulated ($P < 0.05$, quadratic $P < 0.05$) in the liver tissue of the EQs groups compared with that of the CT group, as shown in Table 8.

Effect of dietary supplementation of EQ on gut microbiota community structure of heat-stressed broilers.

The differences in the caecum microbiota among the EQs groups of heat-stressed broilers are shown in Figure 1-3. The results of α-diversity measures for caecum microbiota among groups does not show any significant differences, including Chao1, Simpson, Shannon, Pilelou, Observed species, and Goods coverage (Figure 1A). The results of β-diversity measures for caecum microbiota among groups show significant differences ($p < 0.05$) in principal component analysis (PCA), principal coordinate analysis (PCoA), and non-metric multidimensional scaling (NMDS) analysis of weighted unifrac distance among groups (Figure 1B, D and E). The CT group show a significant correlation in the close cluster by the ANOSIM analysis of PCA, PCoA, and NMDS, far away from the EQs groups of heat-stressed broilers.

The effects of supplementing heat-stressed broilers with different levels of EQ for 5 weeks on the relative abundance of phyla and families (top 20) in the caecum microbiota are shown in Figure 2A and B. At the phylum level, *Firmicutes*, *Proteobacteria*, *Actinobacteriota*, *Bacteroidota*, and *Cyanobacteria* were the predominant (top 5 phyla) in the caecal microbiota (Figure 2A). The analysis at the phylum level revealed that the highest average ($p < 0.05$) relative

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abundance of the *Firmicutes* phyla was 85.83 % and appeared in the EQ-50 group compared to the other groups of the CT (75.18 %), EQ-100 (83.97 %), and EQ-200 (76.93 %). Our results show that there is a significant increase ($p < 0.05$) in the relative abundance of *Bacteroidota* phyla in the EQ-50 group compared to the other groups. However, the lowest average ($p < 0.05$) relative abundance of the *Proteobacteria* phyla from gram-negative bacteria (GNB) was 2.39 %, which appeared also in the EQ-50 group, compared to the other groups of the CT (13.33 %), EQ-100 (12.83 %), and EQ-200 (19.78 %) groups. The highest average ($p < 0.05$) relative abundance of the *Cyanobacteria* phyla (GNB) was 10.61 % which was found in the CT group, and was twenty times as much as that in the other groups: EQ-50 (0.62 %); EQ-100 (0.26 %); and EQ-200 (0.38 %). Likewise, the highest average ($p < 0.05$) relative abundance of the *Actinobacteria* phyla was 9.8 % and was found in the CT group, compared to that of the other groups: EQ-50 (2.74 %), EQ-100 (6.15 %), and EQ-200 (2.77 %). In addition, the relative abundance of *Verrucomicrobia* phyla (GNB) significantly decreased ($p < 0.05$) in the EQ-50 group compared to that of the other groups, as shown in Figure 2. At the genus level, the relative abundance of *Lactobacillus*, *Ligilactobacillus*, *Limosilactobacillus*, *Pediococcus*, *Blautia*, and *Faecalibacterium* were significant increase ($p < 0.05$) in the EQ-50 group, however, the relative abundance of *Streptococcus*, *Enterococcus*, *Escherichia-Shigella*, *Staphylococcus*, *Clostridia*, and *Psychrobacter* were significant increase ($p < 0.05$) in the CT group in compared with other groups (Figure 2A and B).

Effect of dietary supplementation of EQ on gut microbiota community function of heat-stressed broilers.

The results of microbial function prediction by comparing the relative abundance among groups based on the second level of KEGG pathway analysis are shown in Figure 3A. The top 50

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most significant ($p < 0.05$) abundant KEGG orthologous genes were clustered in a heat map, combined with the analysis of microbial function among the groups. All samples from each group formed clusters based on similar microbial compositions. The PCoA plot shows that the samples belonging to the CT group are more similar to each other than those from the EQ groups (Figure 3B). The top five KEGG orthologues annotated in the CT group were lipopolysaccharide-binding protein (K05399), engulfment and cell motility protein 1 (K12366), programmed cell death 1 ligand 2 (K06708), Bcl-2-antagonist of cell death (K02158), and engulfment and cell motility protein 3 (K19241). the top 5 abundance KEGG orthologues annotated in the EQ-50 group were transport system ATP-binding protein (K01990), GTPase (K03595), branched-chain amino acid aminotransferase (K00826), family transcriptional regulator (K03435), and uridine nucleosidase (K01240). Similarly, the top five KEGG orthologues of translation initiation factor (K02518), translation initiation factor (K03113), tricarboxylic transport membrane protein (K07795), arachidonate lipoxygenase (K00460), and basic amino acid family (K03294) were the most annotated in the EQ-100 group. The top five KEGG orthologues of the transport system permease protein (K02033), glycosyltransferase-related protein (K00754), anaerobic regulatory protein (K01420), DNA-repair protein complement (K10847), and RNA-binding transduction-associated protein 3 (K14942) were the most annotated in the EQ-200 group. PCoA analysis revealed significant differences in these KEGG orthologues among the groups (Figure 3B).

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DISCUSSION

In the present study, the addition of 50, 100, and 200 mg EQ/kg diet for five weeks helped prevent OS by enhancing heat tolerance, which induced performance improvements in heat-stressed broilers. This beneficial effect enhances the blood metabolite molecules of hepatic antioxidant enzyme activities and their energy content, promoting serum immunity and cytokine

activities associated with heat tolerance. The effects of EQ on broiler performance have attracted the attention of scientists since the early days of the poultry industry, and exposure of stressed broilers to EQ causes biochemical and physiological antioxidant changes, depending on the administration level (Bartov and Bornstein, 1972; Cabel, et al., 1988; Runnels, et al., 1966). In addition, EQs effectively increase cytokine- and immune-related genes and increase the relative abundance of symbiotic caecum microbiota, attenuating intestinal injury (Tian, Tao, Li and Wang, 2021; Yamashita, et al., 2009). EQ has high antioxidant activity. It is highly efficient at preventing OS in heat-stressed organisms. In animals treated with EQ, three times higher plasma levels of vitamins A and E were observed, suggesting that an organism uses EQ instead of natural antioxidants not only because of the chemical features of EQ itself, but also because the products of its oxidation also possess antioxidative properties (Bartov and Bornstein, 1972; Błaszczyk, Augustyniak and Skolimowski, 2013).

Birds under cyclic HS display reduced intestinal structure (crypt depth, mucous area, and villus height), epithelial cell injury, and hyperpermeability, leading to an influx of bacterial endotoxins (LPS) from the intestinal lumen into the circulatory system and affecting the organ systems (Mishra and Jha, 2019). The LPS or endotoxins induce apoptosis, inflammation, necrosis, and injury in various cell types (Bertani and Ruiz, 2018). Indeed, global climate change associated with HS has more detrimental effects on poultry performance than on mammals owing to the lack of sweat glands, resulting in significant economic loss and raising global concerns for the broiler industry. HS play an important role in physiological disorders, especially intestinal barrier failure, inducing LP, generating ROS, DNA degradation, mitochondrial dysfunction, cell membrane damage, and inactivation of transport enzymes (Biswal, et al., 2022; Calefi, et al., 2017). Heat stress is one of the most common environmental factors affecting the gut health of poultry,

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including physiology, integrity, immunology, and microbiota (Rostagno, 2020). Heat-stressed birds redistribute blood to the periphery to maximise radiant heat dissipation. Consequently, reduced blood and nutrient flow leads to hypoxia in the intestinal epithelium, compromising intestinal integrity. Therefore, many studies have investigated the role of antioxidant compounds, which have facilitated research on the heat tolerance of heat-stressed broilers and the development of new biotherapeutics such as coenzyme Q10, butylated hydroxyanisole (BHA), L-carnitine, L-arginine, ascorbic acid, and alpha-tocopherol (Augustyniak, et al., 2010; Wojcik, et al., 2010). However, these studies did not explore the function of these antioxidant compounds in host-microbiome interactions.

Our results show a significant increase in the final body weight and average daily weight gain in the EQ-50 group, which was caused by promoting mRNA expression of *Hsp70*, *IGF-1*, and *GH* genes in the EQs groups. These results are in agreement with those found by Cabel and Calabotta (1988), who indicated that EQ supplementation at 62.5 and 125 ppm resulted in significantly heavier broiler birds at 49 days of age but had no significant effect on feed efficiency. Significant interactions between the additions of EQ with dl-α-tocopherol acetate to the broiler diet were observed on the blood variables and the activity of glutathione peroxidase in plasma, improving effects on the weight gain and the vitamins profile (Lauridsen, et al., 1995). Similar to our findings, Ohshima et al., (Ohshima, et al., 1996) reported that plasma cholesterol level was significantly reduced from 209 with the control diet to 157 mg dl^{-1} with the diet containing 50 mg kg^{-1} EQ (EQ-50). The inclusion of 2.2% EQ in a commercial canthaxanthin product (CCX) for *in ovo* feeding of broiler embryos shown an improvement in the oxidation status of chicks, the hatchability, and their growth performance (Araújo, et al., 2020). Lauridsen et al. (Lauridsen, et al., 1994) found a significant effect of diet supplementation with 150 ppm EQ on the antioxidative

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and oxidative balance in broilers, which improved weight gain, feed conversion, haematocrit, haemolytic properties of erythrocytes, and plasma activities of the enzymes glutamate oxaloacetate transaminase (GOT), creatine kinase (CK), and glutathione peroxidase (GSH-Px). In laying hens, dietary inclusion of 250 mg EQ/kg $^{-1}$ diet improved egg performance traits and feed efficiency and reduced mortality after an outbreak of Newcastle disease (Bartov, et al., 1991). The primary effect of EQ supplementation (75 ppm) is to enhance the biosynthesis of polyunsaturated fatty acids (PUFA) in broiler livers (Donaldson, 1993), as PUFA deficiency in liver diseases is known to be associated with pathophysiological and clinical significance (Cabré and Gassull, 1996).

Liver antioxidant activity and total adenylate levels were determined to evaluate the health status of heat-stressed broilers supplemented with EQs. The present study shows that diet supplementation with 50 mg EQ/kg^{-1} (EQ-50) can be beneficial for liver antioxidant enzyme activities (GSH, NO, and CAT) and for reducing MDA concentration. However, the liverconcentration of ATP increased in the EQ-100 group, which was caused by the promotion of mRNA expression of *Hsp70*, *SOD2*, *GPx 4*, *IGF-1*, *GH* genes in the EQs groups. For this reason, EQs play a vital role in the biosynthesis and catalytic processes related to cellular antioxidant defence, which regulate candidate roles in the structural and functional enzymes of biological pathways. The observed increase in antioxidant ability was expected because EQ is a well-known feed antioxidant for both domestic animals and fish (Abeyta, et al., 2023; Dai and Cho, 2022). EQ is considered a model inducer of both phase I and II biotransformation enzymes involved in the metabolism of xenobiotics, playing a key role in mediating phase reactions (e.g. oxidation or reduction) and producing more hydrophilic compounds that are catalysed by the CYP (cytochrome P450) enzyme family to enhance the expression of proteins involved in

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detoxification (Berdikova Bohne, et al., 2007). The digestive enzymes, lipase and amylase, were elevated in the EQ-50 group, which correlated with blood antioxidants and immune efficiency. Thus, the weaning administration of milk replacer plus 350 mg EQ/kg^{-1} replacer for 35 days significantly improved the blood activities of GSH-PX, CAT, and T-AOC in dairy Holstein calves (Wei et al., 2023).

The access of chicks to EQ is associated with their immunocompetence and reduced susceptibility to pathogenic infections during their growth period. In the present study, serum IgG, IgM, and IgA levels increased in the EQs group. In addition, serum cytokines profile of IL-6, IL-10, and TNF-α, and TGF-β as similar increased in EQs groups. These findings were caused by promoting mRNA expression of *Hsp70*, *TNFRSF11B*, *TNF*, *IL-6*, and *IL-10* genes in the EQs groups. Indeed, TGF-β regulates a myriad of biological processes during chicken growth, and its disruption often leads to embryonic lethality due to defects in multiple organ systems (Cooley, et al., 2014). Tumour necrosis factor alpha (TNF-α) is expressed in the organogenesis of chicks and regulates the survival, proliferation, and apoptosis of stem cells and progenitor cells (You, et al., 2021). Antioxidant deficiency in heat-stressed broilers leads to lymphatic atrophy, resulting in a decreased response to many T-dependent antigens and reduced plaque-forming cell responsiveness to heterologous red blood cells, similar to what was obtained for the cell function (Ayo and Ogbuagu, 2021; Calefi, et al., 2017). In addition, the overexpression of the IGF-I gene in the liver tissue correlated with lean tissue growth and targeted invalidation of muscle myostatin secretion (Grade, et al., 2019), which was clearly demonstrated in the embryo weight gain in the EQ-50 group.

Broilers are rapidly growing because of the great potential of the intestinal epithelia for nutrient absorption and efficient conversion of nutrients to muscle. Physiologically, ROS and

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reactive nitrogen species (RNS) are generated by gut epithelial cells, either from oxygen metabolism or by enteric gut microbiota, resulting in antioxidant insults that are susceptible to OS. Thus, OS in the poultry gut is derived from chronic HS, which reduces performance by enhancing the opportunistic gut microbiome communities (Mishra and Jha, 2019). However, antioxidant supplements, such as EQ, have ROS scavenger properties that mitigate OS and are beneficial in promoting symbiotic gut microbiome communities.

In the present study, it was found that supplementation with 50, 100, and 200 mg EQ/ kg^{-1} diet for 5 weeks helped prevent intestinal inflammation by enhancing the enteric antioxidant capacity and cytokine secretion, promoting the symbiotic lifestyle of the gut microbiota community in comparison with the control group. Indeed, gut microbiota is emerging as a promising target for the management or prevention of inflammatory and metabolic disorders, including immune responses, the epithelial barrier, and cell proliferation. However, host redox signalling dysfunction due to OS prompts the rapid generation of opportunistic bacteria-induced ROS and RNS such as LPS (Neish and Jones, 2014). Hence, the composition of gut microbiota has been used as a potential biomarker for HS in monogastric species (He, et al., 2021). Optimising host redox signalling through antioxidant supplementation, such as EQ, in heat-stressed broilers may help protect intestinal barrier integrity and symbiotic gut microbiota from OS induced by HS.

Overall, the EQ-50 group showed a greater composition of *Firmicutes* and *Bacteroides* phyla than the other groups, and the potentially useful effects of these bacteria on diet fermentation and digestion led to an increase in small water-soluble food molecules for absorption into watery blood plasma (Elokil, et al., 2020a). The abundance of *Bacteroides,* was lower in the CT group than that in the EQs group, especially in the EQ-50 group. *Bacteroides* produce short-chain fatty acids and are important for the growth of gut microbiota and glucose homeostasis in heat-stressed

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broilers (Shi, et al., 2019). In addition, there are indications that the gut microbiota has a bidirectional relationship with the intestinal system, including its products (such as acetate, propionate, and butyrate from fibre fermentation), and plays a critical role in maintaining the intestinal barrier and integrity functions in the host immune system by promoting immune cell development and differentiation and increasing the secretion of anti-inflammatory cytokines (Cao, et al., 2021; Daniel, et al., 2021).

Our findings show that a distinct microbial community colonised the gut due to EQ supplementation, which improved intestinal function and antimicrobial pathogenicity compared to the CT group. The relative abundances of *Lactobacillus*, *Ligilactobacillus*, *Limosilactobacillus*, *Pediococcus*, *Blautia*, and *Faecalibacterium* increased in the EQ-50 group. These genera are often used as beneficial probiotic products to ameliorate HS by enhancing intestinal barrier function and improving the gut microbiota (Zhang, et al., 2017). Collectively, our results show that the prevalence of intestinal bacterial pathogen phyla (*Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, and *Verrucomicrobia*) in the gut microbiota increased in the CT group of chicks compared to that in the EQ groups. These phyla correlate with the detrimental effects of hyperthermia and OS on the gut epithelium, leading to impaired permeability and susceptibility to infection and inflammation (He, Maltecca and Tiezzi, 2021; Mishra and Jha, 2019). Likewise, the relative abundance of the intestinal bacterial pathogens genus of *Streptococcus*, *Enterococcus*, *Escherichia-Shigella*, *Staphylococcus*, *Clostridia*, and *Psychrobacter* which were raised in the gut microbiota increased in the CT group of chicks. This is in agreement with previous studies which found that enteric bacterial pathogens induced by chronically heat-stressed broilers may cause disturbances in the bile acid pool, leading to lipid metabolism disorders and decreased growth performance (Zhang, et al., 2023). The results of microbial function prediction using second-level

KEGG pathway analysis reveals that the top five KEGG orthologues annotated in the CT group are lipopolysaccharide-binding protein, engulfment and cell motility protein 1, programmed cell death 1 ligand 2, Bcl-2-antagonist of cell death, and engulfment and cell motility protein 3. These functions are closely associated with the OS in heat-stressed broilers (Ayo and Ogbuagu, 2021; Biswal, et al., 2022). In contrast, candidate pathways related to antioxidant defence, such as the transport system ATP-binding protein, GTPase, family transcriptional regulator, and uridine nucleosidase, were highly expressed in the EQ-50 group.

In chicks under heat exposure, there are elevated numbers of several detrimental genera, including *Escherichia*, *Shigella*, and *Clostridium* against advantageous bacteria, such as *Lactobacillus* and *Ruminococcaceae*, leading to the generation of alpha-toxins and contributing to the occurrence of necrotising enterocolitis (Cao, Chowdhury, Cline and Gilbert, 2021). In addition, these genera induce multiple physiological alterations in the microbiota-gut-brain axis of heat-stressed broilers, primarily via bacterially derived metabolites, hormones, and neurotransmitters, which disrupt host metabolic homeostasis, health, and behaviour (Cao, et al., 2021; Zhang, et al., 2023) (Cao, Chowdhury, Cline and Gilbert, 2021; Zhang, Chen, Cong, Zhang, Jia and Wu, 2023). Recently, a mucosal microbiology approach identified some microbial groups such as *Streptococcus*, *Enterococcus*, *Escherichia-Shigella*, *Staphylococcus*, *Clostridia*, and *Psychrobacter* that are closely associated with promoting intestinal inflammatory bowel diseases and it's associated with pathological infection (Daniel, Lécuyer and Chassaing, 2021).

It can be concluded that the improvement in growth performance traits, relative organ index, hepatic antioxidant enzymes, serum immune and cytokine content may be attributed to the prevention of enteric oxidative stress by the abundant antioxidants produced due to the potential ability of EQ supplementation. Dietary supplementation of heat-stressed broilers with 50 mg EQ/

kg^{-1} for 5 wk to heat-stressed broilers enhanced hepatic antioxidant activity (GSH, CAT, and NO) and inhibited MDA production. In addition, it increased the activities of serum IgG, IL-6, and TGF-β, and ultimately enhanced intestinal barrier function for leading to colonise a distinct symbiotic microbiota community such as *Lactobacillus*, *Ligilactobacillus*, *Limosilactobacillus*, and *Faecalibacterium*. Furthermore, EQ promoted the expression of *Hsp70*, *SOD2*, *GPx 4*, *IL-6,* and *IGF-1* cytokine gene-related anti-inflammatory and growth factors in heat-stressed hepatic broilers. Thus, EQ-50 may reduce epithelial cell injury and hyperpermeability, preventing bacterial lipopolysaccharide efflux from the intestinal lumen into the circulatory system and affecting organ systems. In summary, 50 mg EQ kg/diet for five weeks is potentially a suitable feed supplement for attenuating enteric oxidative stress and intestinal inflammation, thus improving productivity in heat-stressed broilers.

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DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

Journal Pre-proof

Abeyta, M., M. Al-Qaisi, E. Horst, E. Mayorga, S. Rodriguez-Jimenez, B. Goetz, S. Carta, H. Tucker, and L. Baumgard. 2023. Effects of dietary antioxidant supplementation on metabolism and inflammatory biomarkers in heat-stressed dairy cows. Journal of Dairy Science 106:1441-1452.

Additives, E. P. o., and P. o. S. u. i. A. Feed. 2015. Safety and efficacy of ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) for all animal species. EFSA Journal 13:4272. doi https://doi.org/10.2903/j.efsa.2015.4272

Araújo, I. C., M. B. Café, M. A. Mesquita, B. N. Caiado, A. M. Faria, H. H. Mello, J. H. Stringhini, and N. S. Leandro. 2020. Effect of a commercial product containing canthaxanthin for in ovo feeding to broiler embryos on hatchability, chick quality, oxidation status, and performance. Poultry science 99:5598-5606.

Augustyniak, A., G. Bartosz, A. Čipak, G. Duburs, L. U. Horáková, W. Łuczaj, M. Majekova, A. D. Odysseos, L. Rackova, and E. Skrzydlewska. 2010. Natural and synthetic antioxidants: an updated overview. Free radical research 44:1216-1262.

Ayo, J., and N. Ogbuagu. 2021. Heat stress, haematology and small intestinal morphology in broiler chickens: insight into impact and antioxidant-induced amelioration. World's Poultry Science Journal 77:949-968.

Bartov, I., and S. Bornstein. 1972. Comparisons of BHT and Ethoxyquin as Antioxidants in the Nutrition of Broilers1. Poultry Science 51:859-868. doi https://doi.org/10.3382/ps.0510859

Bartov, I., Y. Weisman, and E. Wax. 1991. Effects of high concentrations of dietary vitamin e and ethoxyquin on the performance of laying hens1. British Poultry Science 32:525-534. doi 10.1080/00071669108417377

Berdikova Bohne, V. J., K. Hamre, and A. Arukwe. 2007. Hepatic metabolism, phase I and II biotransformation enzymes in Atlantic salmon (Salmo Salar, L) during a 12 week feeding period with graded levels of the synthetic antioxidant, ethoxyquin. Food and Chemical Toxicology 45:733-746. doi https://doi.org/10.1016/j.fct.2006.10.020

Bernhard, A., J. D. Rasinger, M. B. Betancor, M. J. Caballero, M. H. Berntssen, A.-K. Lundebye, and R. Ørnsrud. 2019. Tolerance and dose-response assessment of subchronic dietary ethoxyquin exposure in Atlantic salmon (Salmo salar L.). PloS one 14:e0211128.

Bertani, B., and N. Ruiz. 2018. Function and Biogenesis of Lipopolysaccharides. EcoSal Plus 8. doi 10.1128/ecosalplus.ESP-0001-2018

Biswal, J., K. Vijayalakshmy, B. T. K, and H. Rahman. 2022. Impact of heat stress on poultry production. World's Poultry Science Journal 78:179-196.

Błaszczyk, A., A. Augustyniak, and J. Skolimowski. 2013. Ethoxyquin: An Antioxidant Used in Animal Feed. International Journal of Food Science 2013:585931. doi 10.1155/2013/585931

Cabel, M. C., P. W. Waldroup, W. D. Shermer, and D. F. Calabotta. 1988. Effects of Ethoxyquin Feed Preservative and Peroxide Level on Broiler Performance1. Poultry Science 67:1725-1730. doi https://doi.org/10.3382/ps.0671725

Journal Pre-proof

Cabré, E., and M. A. Gassull. 1996. Polyunsaturated fatty acid deficiency in liver diseases: pathophysiological and clinical significance. Nutrition (Burbank, Los Angeles County, Calif.) 12:542-548. doi 10.1016/s0899-9007(96)00122-0

Calefi, A. S., W. M. QUINTEIRO-FILHO, A. J. P. Ferreira, and J. Palermo-Neto. 2017. Neuroimmunomodulation and heat stress in poultry. World's Poultry Science Journal 73:493-504.

Cao, C., V. S. Chowdhury, M. A. Cline, and E. R. Gilbert. 2021. The Microbiota-Gut-Brain Axis During Heat Stress in Chickens: A Review. Front Physiol 12:752265. doi 10.3389/fphys.2021.752265

Chen, J., F. Li, W. Yang, S. Jiang, and Y. Li. 2021. Supplementation with exogenous catalase from Penicillium notatum in the diet ameliorates lipopolysaccharide-induced intestinal oxidative damage through affecting intestinal antioxidant capacity and microbiota in weaned pigs. Microbiology Spectrum 9:e00654-00621.

Cooley, J. R., T. A. Yatskievych, and P. B. Antin. 2014. Embryonic expression of the transforming growth factor beta ligand and receptor genes in chicken. Developmental Dynamics 243:497-508. doi https://doi.org/10.1002/dvdy.24085

Dai, Q., and S. H. Cho. 2022. Dietary inclusion effect of citrus peel by-product as an additive on the growth performance, body composition, and various stress resistance of juvenile abalone (Haliotis discus) compared to ethoxyquin. Aquaculture Reports 24:101187.

Daniel, N., E. Lécuyer, and B. Chassaing. 2021. Host/microbiota interactions in health and diseases-Time for mucosal microbiology! Mucosal immunology 14:1006-1016. doi 10.1038/s41385-021-00383-w

de Koning, A. J. 2002. The antioxidant ethoxyquin and its analogues: a review. International Journal of Food Properties 5:451-461.

Donaldson, W. 1993. Effects of dietary lead, fish oil, and ethoxyquin on hepatic fatty acid composition in chicks. Biological trace element research 36:319-326.

Elokil, A., M. Magdy, S. Melak, H. Ishfaq, A. Bhuiyan, L. Cui, M. Jamil, S. Zhao, and S. Li. 2020a. Faecal microbiome sequences in relation to the egg-laying performance of hens using amplicon-based metagenomic association analysis. animal 14:706-715.

Elokil, A. A., K. Abouelezz, A. A. Adetula, H. I. Ahmad, C. Mo, C. Sun, and S. Li. 2020b. Investigation of the impact of gut microbiotas on fertility of stored sperm by types of hens. Poultry Science 99:1174-1184.

Elokil, A. A., K. F. Abouelezz, H. I. Ahmad, Y. Pan, and S. Li. 2020c. Investigation of the Impacts of Antibiotic Exposure on the Diversity of the Gut Microbiota in Chicks. Animals 10:896.

Elokil, A. A., W. Chen, K. Mahrose, M. M. Elattrouny, K. F. M. Abouelezz, H. I. Ahmad, H.-Z. Liu, A. A. Elolimy, M. I. Mandouh, A. M. Abdelatty, and S. Li. 2022. Early life microbiota transplantation from highly feed-efficient broiler improved weight gain by reshaping the gut microbiota in laying chicken. Frontiers in Microbiology 13. doi 10.3389/fmicb.2022.1022783

Journal Pre-proof

Journal Pre-proof

Gai, X., D. He, W. Yang, M. Han, W. Lai, and L. Zhang. 2018. Effects of high dose of ethoxyquin on growth performance, serum biochemical indices, antioxidant capacity, organ index and meat quality of growing-finishing pigs. Chinese Journal of Animal Nutrition 30:3643-3651.

Grade, C. V. C., C. S. Mantovani, and L. E. Alvares. 2019. Myostatin gene promoter: structure, conservation and importance as a target for muscle modulation. Journal of animal science and biotechnology 10:1-19.

He, Y., C. Maltecca, and F. Tiezzi. 2021. Potential Use of Gut Microbiota Composition as a Biomarker of Heat Stress in Monogastric Species: A Review. Animals 11:1833.

Iskusnykh, I. Y., E. D. Kryl'skii, D. A. Brazhnikova, T. N. Popova, K. S. Shikhaliev, K. K. Shulgin, L. V. Matasova, S. S. Popov, D. A. Zhaglin, and A. A. Zakharova. 2021. Novel antioxidant, deethylated ethoxyquin, protects against carbon tetrachloride induced hepatotoxicity in rats by inhibiting NLRP3 inflammasome activation and apoptosis. Antioxidants 10:122.

Kuttappan, V. A., M. Manangi, M. Bekker, J. Chen, and M. Vazquez-Anon. 2021. Nutritional Intervention Strategies Using Dietary Antioxidants and Organic Trace Minerals to Reduce the Incidence of Wooden Breast and Other Carcass Quality Defects in Broiler Birds. Frontiers in Physiology 12. doi 10.3389/fphys.2021.663409

Lauridsen, C., K. Jakobsen, and T. K. Hansen. 1995. The influence of dietary ethoxyquin on the vitamin E status in broilers. Archiv für Tierernaehrung 47:245-254. doi 10.1080/17450399509381809

Lauridsen, C., K. Jakobsen, and J. F. Jensen. 1994. Comparative studies on the effect of butylhydroxytoluene and ethoxyquin on the antioxidative and oxidative balance in broilers. Journal of Animal Physiology and Animal Nutrition 72:26-37. doi https://doi.org/10.1111/j.1439-0396.1994.tb00367.x

Li, H., S. Tang, W. Du, J. Jiang, P. Peng, P. Yuan, Y. Liao, J. Long, and S. Zhou. 2019. The effects of ethoxyquin and Angelica sinensis extracts on lipid oxidation in fish feeds and growth, digestive and absorptive capacities and antioxidant status in juvenile red carp (Cyprinus carpio var. xingguonensis): a comparative study. Fish physiology and biochemistry 45:43-61.

Mishra, B., and R. Jha. 2019. Oxidative Stress in the Poultry Gut: Potential Challenges and Interventions. Frontiers in Veterinary Science 6. doi 10.3389/fvets.2019.00060

Neish, A. S., and R. M. Jones. 2014. Redox signaling mediates symbiosis between the gut microbiota and the intestine. Gut microbes 5:250-253. doi 10.4161/gmic.27917

Ohshima, M., D. V. Layug, H.-o. Yokota, and H. T. Ostrowski-Meissner. 1996. Effect of graded levels of ethoxyquin in alfalfa leaf extracts on carotenoid and cholesterol concentrations in chicks. Animal Feed Science and Technology 62:141-150. doi https://doi.org/10.1016/S0377-8401(96)00969-8

Pradhan, A., C. Bereketoglu, L. Martin, J. Duhagon, and P.-E. Olsson. 2020. The food preservative ethoxyquin impairs zebrafish development, behavior and alters gene expression profile. Food and Chemical Toxicology 135:110926.

Rostagno, M. H. 2020. Effects of heat stress on the gut health of poultry. Journal of Animal Science 98:skaa090.

Journal Pre-proof

Runnels, T., B. Kobrin, and C. Ely. 1966. The relative effect of ethoxyquin versus BHT on broiler performance and pigmentation. Poultry Science 45:1283-1290.

Sahoo, D. K., R. M. Heilmann, B. Paital, A. Patel, V. K. Yadav, D. Wong, and A. E. Jergens. 2023. Oxidative stress, hormones, and effects of natural antioxidants on intestinal inflammation in inflammatory bowel disease. Frontiers in Endocrinology 14. doi 10.3389/fendo.2023.1217165

Shi, D., L. Bai, Q. Qu, S. Zhou, M. Yang, S. Guo, Q. Li, and C. Liu. 2019. Impact of gut microbiota structure in heat-stressed broilers. Poultry Science 98:2405-2413. doi https://doi.org/10.3382/ps/pez026

Tadokoro, T., M. Ikeda, K. Abe, T. Ide, H. D. Miyamoto, S. Furusawa, K. Ishimaru, M. Watanabe, A. Ishikita, and S. Matsushima. 2022. Ethoxyquin is a competent radical-trapping antioxidant for preventing ferroptosis in doxorubicin cardiotoxicity. Journal of Cardiovascular Pharmacology 80:690-699.

Tang, D., J. Wu, H. Jiao, X. Wang, J. Zhao, and H. Lin. 2019. The development of antioxidant system in the intestinal tract of broiler chickens. Poultry science 98:664-678.

Tian, J., Q. Tao, Y. Li, and G. Wang. 2021. Ethoxyquin attenuate oxidant stress, inflammatory response and apoptosis in liver of Channa argus fed with high-fat dietary. Aquaculture Reports 21:100889.

Wang, C., J. Liu, B. Duan, Y. Lao, P. X. Qi, and D. Ren. 2018. Effects of dietary antioxidant supplementation of feed, milk processing and storage on the lutein content and sensory quality of bovine milk. International Journal of Dairy Technology 71:849-856. doi https://doi.org/10.1111/1471-0307.12532

Wang, J., Q. Ai, K. Mai, H. Xu, R. Zuo, W. Xu, W. Zhang, and C. Zhang. 2015. Effects of dietary ethoxyquin on growth, feed utilization and residue in the muscle of juvenile Japanese seabass, Lateolabrax japonicus. Aquaculture Research 46:2656-2664.

Wei, X., J. Zou, Y. Zhang, J. Yang, J. Wang, Y. Wang, and C. Wang. 2023. Effects of milk, milk replacer, and milk replacer plus ethoxyquin on the growth performance, weaning stress, and the fecal microbiota of Holstein dairy calves. Frontiers in Microbiology 14:1113518.

Wojcik, M., I. Burzynska-Pedziwiatr, and L. Wozniak. 2010. A review of natural and synthetic antioxidants important for health and longevity. Current Medicinal Chemistry 17:3262-3288.

Yamashita, Y., T. Katagiri, N. Pirarat, K. Futami, M. Endo, and M. Maita. 2009. The synthetic antioxidant, ethoxyquin, adversely affects immunity in tilapia (Oreochromis niloticus). Aquaculture Nutrition 15:144-151.

You, K., H. Gu, Z. Yuan, and X. Xu. 2021. Tumor Necrosis Factor Alpha Signaling and Organogenesis. Frontiers in Cell and Developmental Biology 9. doi 10.3389/fcell.2021.727075

Zhang, P., T. Yan, X. Wang, S. Kuang, Y. Xiao, W. Lu, and D. Bi. 2017. Probiotic mixture ameliorates heat stress of laying hens by enhancing intestinal barrier function and improving gut microbiota. Italian Journal of Animal Science 16:292-300. doi 10.1080/1828051X.2016.1264261

Journal Pre-proof

Zhang, Y., H. Chen, W. Cong, K. Zhang, Y. Jia, and L. Wu. 2023. Chronic Heat Stress Affects Bile Acid Profile and Gut Microbiota in Broilers. Int J Mol Sci 24. doi 10.3390/ijms241210238

Table 1. Ingredients and calculated nutrient levels of starter, grower and finisher of the basal diet (air-dry basis, %).

Ingredients	Starter (0-14 days)	Grower (15-28 days)	Finisher (29-35 days)
Yellow corn	542	558.8	606
Soybean meal (44%)	319	281	253.3
Corn gluten meal (60%)	71	81	48.1
Vegetable oil	29.8	41	54.4
Limestone [1]	15	15	15
Monocalcium phosphate [2]	14	14	14
Common salt	3	3	3
Mineral Premix [3]	1.5	1.5	1.5
Vitamin Premix[3]	1.5	1.5	1.5
Methionine [4]	1	1	1
Lysine[5]	1	1	1
Anti-coccidial[6]	0.2	0.2	0.2
Antimould 7	1	1	1
Calculated analysis			
Crude protein (CP, %)	23.1	22.18	19.39
Metabolizable energy (ME, Kcal/kg diet)	3053	3160.7	3252.6
Calorie/protein ratio	132.6	142.5	167.7

1 Limestone contains 36% calcium. 2 Monocalcium phosphate contains 22% phosphorus and 16% calcium. 3 Mineral and Vitamin premix was produced by Heropharm and composed (per 3 kg) of vitamin A 12,000,000 IU, vitamin D3 2,500,000 IU, vitamin E 15,000 IU, vitamin K3 1000 mg, vitamin B1 1000 mg, vitamin B2 3000 mg, vitamin B6 1500 mg, vitamin B12 13.3 mg, niacin 30,000mg, biotin 50 mg, folic acid 600 mg, pantothenic acid 10,000 mg, Mn: 60,000 mg, Zn: 50,000 mg, Fe: 30,000 mg, Cu: 4000 mg, I: 300 mg, Se: 100 mg and Co:100 mg. 4 DL-Methionine (Produced by Evonic Co. Birmingham, AL,

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Germany and contain 99% methionine), 5 Lysine (lysine hydrochloride contains 98 % Lysine). 6 Kill Cox® (produced by Arabian Company for Pharmaceutical Industries, 7 Antimould produced by EL-TOBA CO. For Premixes & Feed El-Sadat city Egypt).

Table 2. Primer sequences used for the study of gene expression of selected targets by quantitative real-time PCR.

Gene[1]	Primer sequences (5′ to 3′)	Annealing temperature, °C	PCR product size, bp	Accession No.
GH	F-TCAAGCAACACCTGAGCAAC R-CTTTGTATGTCTCGGCAGCC	61	131	XM_015885521.2
Hsp70	F:- GCTAAGGGCGAGGTCTACAT R:-TCTTCAGCTGCAGATCGTCT	60	145	XM_015856892.2
IGF-1	F-CTTGTGGATGGCATGATCT R-CACCTAAATCTGCACGCT	60	174	XM_015867574.2
SOD2	F-CAGAATGTTGACCCCTCCTACC R-CTATCGACACAGCCTGCTCCT	60	122	XM_015871200.2
GPx 4	F-TCT*AAGCTT* CGGAGAATGTGCGCTCA R-CG*GAATTC*GACAGCAGATACTACATTA	62	134	NM_001277853.1
MUC2	F-AATGCTGAGTTCTTGCCTAA R-TGTTGCAGTTCATATCCTGGT	60	153	XM_001234581.3
TNFRSF11B	F-TCCACCGAGACCGGACTG R-ACAAAACTTTCGCTTGCT	62	238	NM_000164761.1
Il-6	F-CAAGGTGACGGAGGAGGAC R-TGGCGAGGAGGGATTTCT	60	127	NM_204628
Il-10	F-CAGCACCAGTCATCAGCAGAGC R-GCAGGTGAAGAAGCGGTGACAG	61	132	NM_001004414.2
β-actin	F-CGCAAGTACTCGGTGTGGAT R-CCGACTCGTCATACTCCTGC	60	101	NM_001101683.1

[1] F = forward; R = reverse; *GH* = growth hormone; *Hsp70* = heat shock protein 70; IGF-1 = insulin-like growth factor 1; *SOD2* = superoxide dismutase 2; *GPx 4* = glutathione peroxidase; *MUC2* = mucin 2; *TNFRSF11B* = TNF receptor superfamily member 11b; *Il-6* = interleukin 6; *Il-10* = interleukin 10; *β-actin* = beta-actin.

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Table 3. Effect of dietary supplementation of Ethoxyquin (EQ) on the growth performance of heat-stressed broilers.

Variables[1]	Ethoxyquin (EQ) supplementation[2]				SEM	*P*-value[3]		
	CT	EQ-50	EQ-100	EQ-200		P_{ANOVA}	P_{lin}	P_{quad}
IBW (g)	44.90	44.50	44.80	44.90	3.215	0.078	0.114	0.104
FBW (g)	2207.20[b]	2318.50[a]	2282.80[ab]	2307.30[a]	13.541	0.002	0.003	0.016
ADFI (g/day)	87.37[b]	93.21[a]	91.31[a]	88.77[b]	1.304	0.036	0.528	0.673
ADG (g/day)	61.20[b]	65.00 [a]	63.90[a]	64.70[a]	1.271	0.031	0.021	0.015
FCR (g/g)	1.41[a]	1.44[a]	1.42[a]	1.31[b]	0.042	0.028	0.117	0.109
Survivability (%)	90.13[b]	96.67[a]	90.00[b]	96.25[a]	2.431	0.041	0.745	0.209

[1]IBW = initial body weight at 1st day of age; FBW = final body weight at 35th day of age, ADG = average daily body gain; ADFI = average daily feed intake; FCR = feed conversion ratio (g/g), ADFI (g/day)/ADG (g/day).

[2]All data are expressed as mean ± SEM (n = 50 chicks/group). Means in the same row within each classification bearing different letters superscripts are significantly (P≤0.05) different. CT, EQ-50, EQ-100 and EQ-200 = chicks were fed basal diet supplemented with 0mg, 50mg, 100mg and 200mg EQ/kg diet, respectively.

[3] The statistical analysis tests the differences between EQs treatments (ANOVA) and the linear (lin) and quadratic (quad) effect of EQ inclusion levels (polynomial contrasts). SEM= pooled standard error of mean.

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Table 4. Effect of dietary supplementation of Ethoxyquin (EQ) on carcass and relative organ index of heat-stressed broilers.

Variables[1]	Ethoxyquin (EQ) supplementation[2]				SEM	P-value[3]		
	CT	EQ-50	EQ-100	EQ-200		P_{ANOVA}	P_{lin}	P_{quad}
Live body weight (g)	2344.14[b]	2363.32[b]	2434.13[a]	2432.51[a]	16.422	0.001	0.006	0.001
Carcass yield (g)	1707.07[b]	1738.13[b]	1785.05[a]	1766.02[a]	7.255	0.043	0.032	0.046
Dressing, %	73.03	74.01	73.02	73.05	3.250	0.344	0.489	0.351
Head, %	2.67	2.65	2.67	2.58	0.038	0.560	0.194	0.288
Leg, %	3.69	3.63	3.95	3.66	0.057	0.256	0.283	0.681
Gizzard, %	2.01[a]	1.91[b]	1.87[b]	1.80[b]	0.024	0.045	0.083	0.146
Liver, %	2.28[b]	2.08[b]	2.29[b]	2.43[a]	0.029	0.021	0.283	0.461
Spleen, %	0.50	0.49	0.56	0.51	0.021	0.101	0.115	0.083
Heart, %	0.39[b]	0.39[b]	0.63 [a]	0.34[b]	0.020	0.031	0.0372	0.049
Intestine, %	5.85	6.06	5.17	5.73	0.366	0.451	0.120	0.143

[1]The relative organ index = organ weight/living weight × 100%.

[2]All data are expressed as mean ± SEM (n = 20 chicks/group). Means in the same row within each classification bearing different letters superscripts are significantly (P≤0.05) different. CT, EQ-50, EQ-100 and EQ-200 = chicks were fed basal diet supplemented with 0mg, 50mg, 100mg and 200mg EQ/kg diet, respectively.

[3] The statistical analysis tests the differences between EQs treatments (ANOVA) and the linear (lin) and quadratic (quad) effect of EQ inclusion levels (polynomial contrasts). SEM= pooled standard error of mean.

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Table 5. Effect of dietary supplementation of Ethoxyquin (EQ) on hepatic antioxidant enzymes activities and total adenylate in heat-stressed broilers.

Variables[1]	Ethoxyquin (EQ) supplementation[2]				SEM	P-value[3]		
	CT	EQ-50	EQ-100	EQ-200		P_{ANOVA}	P_{lin}	P_{quad}
Liver tissue								
SOD (µmol/g)	52.08[b]	72.42[a]	74.06[a]	54.38[b]	4.658	0.007	0.033	0.042
MDA (nmol/g)	33.14[a]	22.64[b]	24.95[b]	24.92[b]	3.364	0.021	0.034	0.068
GSH (µmol/g)	3.37	4.51	4.43	3.17	0.478	0.143	0.115	0.204
GSSG (nmol/g)	0.33	0.29	0.25	0.32	0.032	0.176	0.781	0.552
NO (µmol/g)	0.31[b]	0.46[a]	0.44[a]	0.32[b]	0.039	0.025	0.046	0.034
CAT (µmol/g)	23.46[ab]	29.62[a]	27.23[a]	12.64[b]	2.948	0.033	0.511	0.489
ATP (µmol/g)	78.24[b]	118.14[a]	119.82[a]	83.63[b]	9.325	0.017	0.029	0.661
ADP (µmol/g)	34.81[a]	25.13[b]	24.26[b]	34.24[a]	3.025	0.035	0.078	0.125
AMP (nmol/g)	17.13[a]	11.06[b]	12.14[b]	17.28[a]	1.653	0.029	0.167	0.049
Intestine mucosa								
Lipase (nmol/g)	65.61[b]	96.26[a]	95.62[a]	68.88[b]	8.299	0.026	0.081	0.493
Amylase (nmol/g)	32.84[b]	50.02[a]	48.78[a]	35.14[b]	4.594	0.031	0.560	0.512

[1]SOD = superoxide dismutase; MDA = malondialdehyde; GSH = glutathione; GSSG = glutathione disulfide; NO = nitric oxide; CAT = catalase; ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenosine monophosphate.

[2]All data are expressed as mean ± SEM (n = 10 chicks/group). Means in the same row within each classification bearing different letters superscripts are significantly (P≤0.05) different. CT, EQ-50, EQ-100 and EQ-200 = chicks were fed basal diet supplemented with 0mg, 50mg, 100mg and 200mg EQ/kg diet, respectively.

[3] The statistical analysis tests the differences between EQs treatments (ANOVA) and the linear (lin) and quadratic (quad) effect of EQ inclusion levels (polynomial contrasts). SEM= pooled standard error of mean.

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Table 6. Effect of dietary supplementation of Ethoxyquin (EQ) on serum immunity and cytokine activities in heat-stressed broilers.

Variables[1]	Ethoxyquin (EQ) supplementation[2]				SEM	P-value[3]		
	CT	EQ-50	EQ-100	EQ-200		P_{ANOVA}	P_{lin}	P_{quad}
IgG (ug/ml)	0.91[b]	1.46[a]	0.92[b]	1.08[b]	0.086	0.011	0.015	0.063
IgA (ug/ml)	0.31[b]	0.48[a]	0.52[a]	0.42[ab]	0.064	0.046	0.193	0.211
IgM (ug/ml)	0.51[b]	0.56[b]	0.86[a]	0.96[a]	0.141	0.001	0.192	0.001
ALT (nmol/ml)	31.19[a]	22.62[b]	22.31[b]	30.87[a]	3.701	0.017	0.191	0.086
AST (nmol/ml)	21.61[a]	14.85[b]	15.23[b]	22.42[a]	2.052	0.028	0.034	0.191
IL-10 (ng/L)	3.49[b]	4.61[a]	4.66[a]	3.20[b]	0.446	0.050	0.266	0.128
IL-6 (ng/L)	13.09[b]	14.89[a]	14.72[a]	13.38[b]	0.548	0.051	0.183	0.175
TNF-α (ng/L)	64.09[b]	69.72[b]	75.89[a]	68.38[b]	2.234	0.024	0.030	0.072
TGF-β (ng/L)	135.52[b]	156.27[a]	151.46[a]	137.68[b]	5.427	0.035	0.021	0.193

[1]IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; TGF-β = transforming growth factor beta; TNF-α = tumor necrosis factor-alpha; IL10 = interleukin 10; IL6 = interleukin 6.

[2]All data are expressed as mean ± SEM (n = 10 chicks/group). Means in the same row within each classification bearing different letters superscripts are significantly ($P \leq 0.05$) different. CT, EQ-50, EQ-100 and EQ-200 = chicks were fed basal diet supplemented with 0mg, 50mg, 100mg and 200mg EQ/kg diet, respectively.

[3] The statistical analysis tests the differences between EQs treatments (ANOVA) and the linear (lin) and quadratic (quad) effect of EQ inclusion levels (polynomial contrasts). SEM= pooled standard error of mean.

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Table 7. Effect of dietary supplementation of Ethoxyquin (EQ) on the essential amino acid profile in MLD basis on dry matter in heat-stressed broilers.

Variables[1]	Ethoxyquin (EQ) supplementation[2]				SEM	*P*-value[3]		
	CT	EQ-50	EQ-100	EQ-200		P_{ANOVA}	P_{lin}	P_{quad}
Lysine	12.28[a]	8.13[b]	8.95[b]	12.82[a]	1.221	0.034	0.104	0.122
Leucine	8.55[b]	12.35[a]	12.07[a]	9.04[b]	0.975	0.024	0.044	0.081
Isoleucine	5.06[b]	5.51[b]	7.11[a]	7.62[a]	0.502	0.050	0.456	0.172
Valine	5.49	6.44	6.50	5.67	0.921	0.131	0.062	0.117
Methionine	2.15[b]	2.87[a]	2.96[a]	2.18[b]	0.237	0.046	0.064	0.108
Serine	8.93[b]	13.99[a]	13.36[a]	10.01[b]	1.221	0.059	0.419	0.338
Aspartic	25.41[a]	24.93[a]	17.34[b]	19.21[b]	2.028	0.021	0.064	0.048
Glutamic	5.19[b]	7.76[a]	7.51[a]	5.21[b]	0.674	0.026	0.186	0.101
Alanine	8.66[a]	5.27[b]	7.68[a]	5.19[b]	0.746	0.035	0.039	0.022
Arginine	4.04[b]	5.97[a]	6.07[a]	4.51[b]	0.331	0.001	0.001	0.043
Histidine	4.96	5.62	5.82	5.36	0.802	0.172	0.276	0.119
Glycine	4.12[b]	4.35[b]	5.84[a]	6.25[a]	0.527	0.025	0.118	0.202
Tyrosine	3.09	3.66	3.61	3.22	0.398	0.161	0.227	0.531
Threonine	5.94[a]	5.26[a]	5.82[a]	4.31[b]	0.246	0.032	0.004	0.018

[1] Muscle amino acids content per mmol/g tissue.

[2]All data are expressed as mean ± SEM (n = 10 chicks/group). Means in the same row within each classification bearing different letters superscripts are significantly (P≤0.05) different. CT, EQ-50, EQ-100 and EQ-200 = chicks were fed basal diet supplemented with 0mg, 50mg, 100mg and 200mg EQ/kg diet, respectively.

[3] The statistical analysis tests the differences between EQs treatments (ANOVA) and the linear (lin) and quadratic (quad) effect of EQ inclusion levels (polynomial contrasts). SEM= pooled standard error of mean.

Table 8. Effect of dietary supplementation of Ethoxyquin (EQ) on hepatic cytokines gene-related anti-inflammatory and growth factors in heat-stressed broilers.

Gene name[1]	Ethoxyquin (EQ) supplementation[2]				SEM	*P*-value[3]		
	CT	EQ-50	EQ-100	EQ-200		P_{ANOVA}	P_{lin}	P_{quad}
Hsp70	1.03 [b]	1.47 [b]	1.60 [a]	1.67 [a]	0.021	0.011	0.023	0.016
SOD2	1.01 [b]	1.34 [b]	1.75 [a]	1.59 [a]	0.033	0.046	0.116	0.205
GPx 4	1.07 [b]	1.71 [a]	1.54 [b]	1.68 [a]	0.027	0.001	0.019	0.107
IGF-1	1.00 [b]	1.88 [a]	1.65 [b]	1.82 [a]	0.038	0.017	0.115	0.163
GH	1.02 [b]	1.66 [b]	1.73 [a]	1.69 [a]	0.026	0.028	0.108	0.096
MUC2	1.00 [b]	1.94 [a]	1.87 [a]	1.55 [b]	0.026	0.071	0.293	0.178
TNFRSF11B	1.01 [b]	1.49 [b]	1.64 [a]	1.66 [a]	0.029	0.052	0.234	0.178
Il-6	1.03 [b]	1.99 [a]	1.67 [b]	1.92 [a]	0.034	0.024	0.063	0.084
Il-10	1.04 [a]	0.57 [b]	0.40 [b]	0.46 [b]	0.022	0.035	0.055	0.047

[1]*Hsp70* = heat shock protein family A; *SOD2* = superoxide dismutase 2, mitochondrial; *GPx 4* = glutathione peroxidase 4; *IGF-1* = insulin-like growth factor 1; *GH* = growth hormone; *MUC2* = mucin 2; *TNFRSF11B* = TNF receptor superfamily member 11b; *Il-6* = interleukin 6; *Il-10* = interleukin 10. The mRNA expression levels were calculated as the relative quantities (RQ) of the target gene mRNA to the geometric mean of β-actin mRNA using the $2^{-\triangle\triangle Ct}$ method.

[2]All data are expressed as mean ± SEM (n = 5 chicks/group). Means in the same row within each classification bearing different letters superscripts are significantly (P≤0.05) different. CT, EQ-50, EQ-100 and EQ-200 = chicks were fed basal diet supplemented with 0mg, 50mg, 100mg and 200mg EQ/kg diet, respectively.

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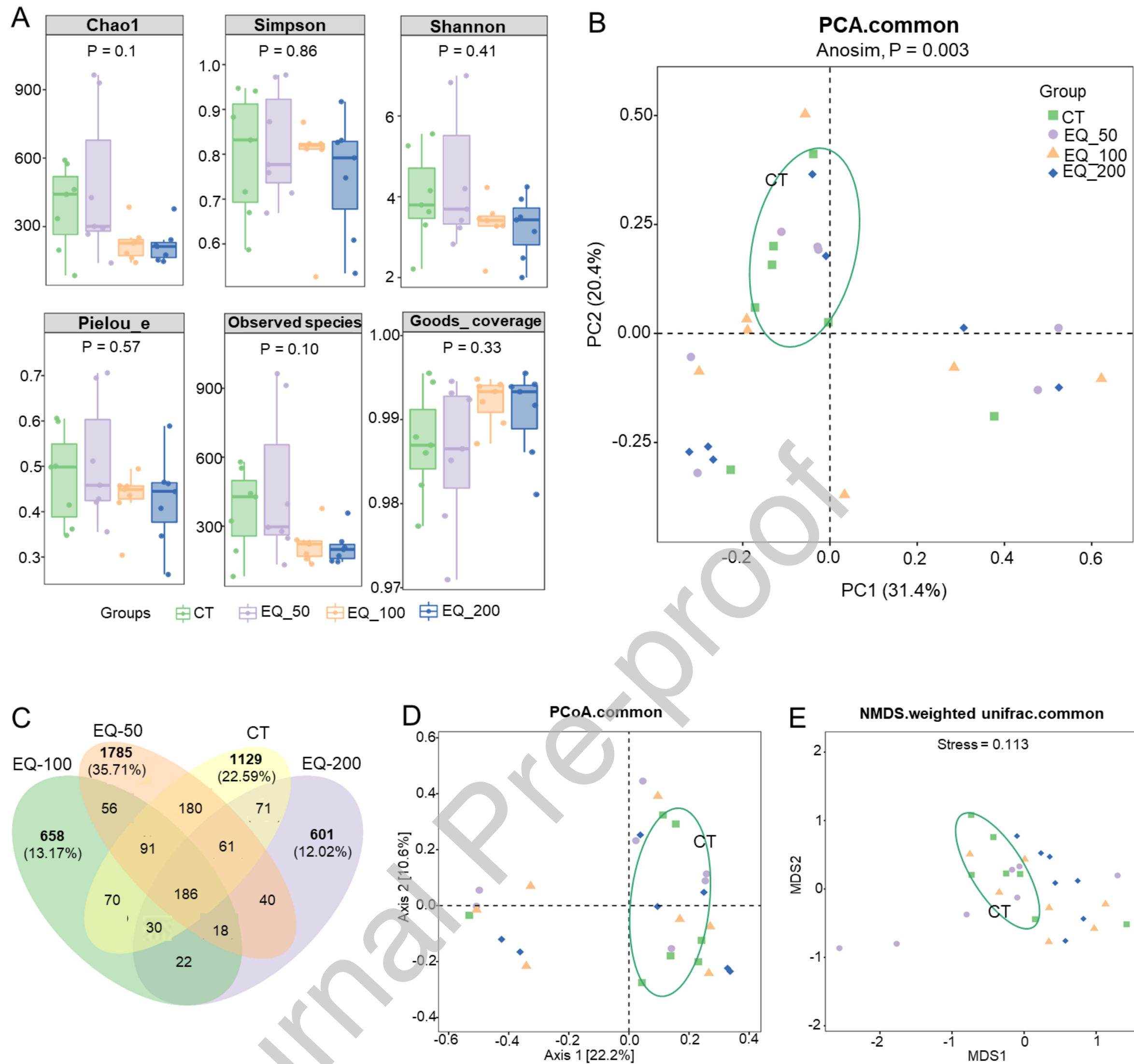

Figure 1. (A) α-diversity measures of bacterial communities among groups of Ethoxyquin (EQ) supplementation in heat-stressed broilers; (B) The PCA (principal component analysis) based on unweighted UniFrac distance. (C) Venn diagram of OUT number among groups of common and unique units. (D Principal coordinate analysis (PCoA) among groups. (E) non-metric multidimensional scaling (NMDS) analysis of weighted unifrac distance among groups.

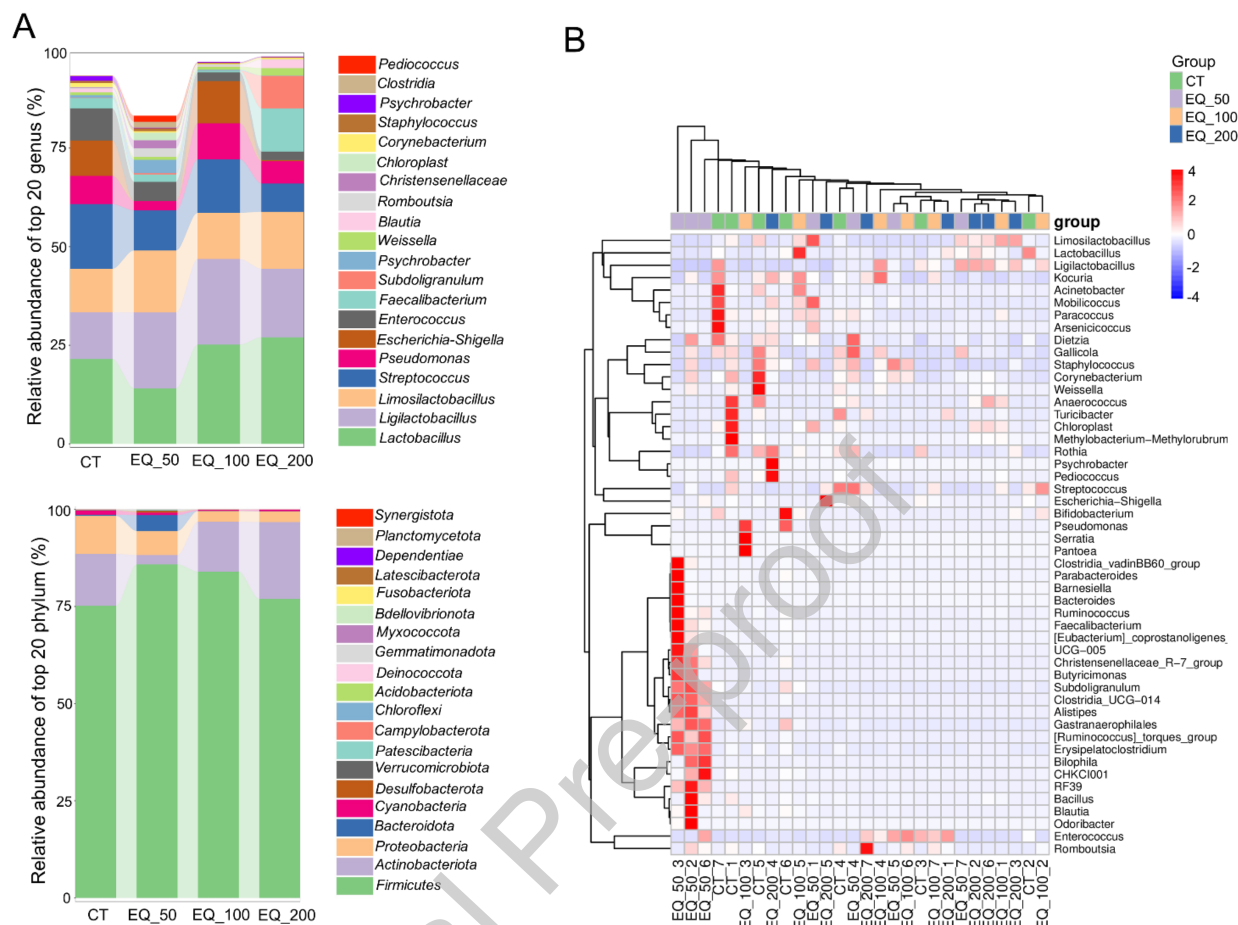

Figure 2. Bacterial community structure among groups of Ethoxyquin (EQ) supplementation in heat-stressed broilers. (A) Taxonomic composition analysis at each classification level of phylum and genus of bacterial communities (B) Heat map showing the genera with significant differences of relative abundances among groups.

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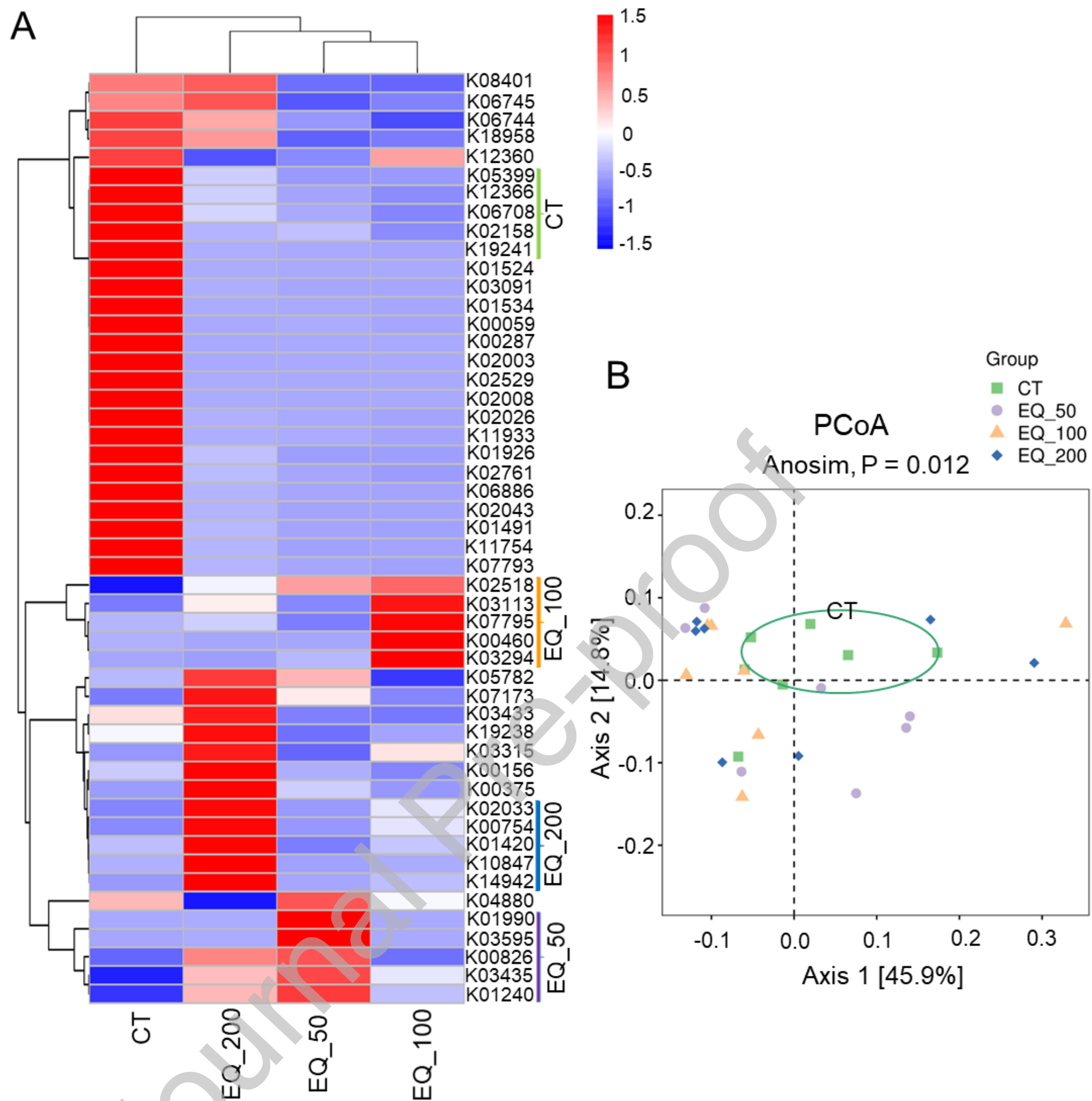

Figure 3. (A) KEGG orthologous gene cluster abundance heat map combined with cluster analysis based on the similarity of the functional group abundance distribution between samples (red represents a functional group with higher abundance in the corresponding sample, and blue represents a functional group with lower abundance) among groups of Ethoxyquin (EQ) supplementation in heat-stressed broilers; (B) Principal coordinate analysis (PCoA) of KEGG orthologous among groups.

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Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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