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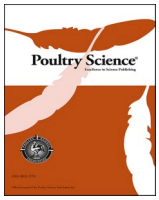

Bamboo leaf flavonoids ameliorate cyclic heat stress-induced oxidative damage in broiler liver through activation of Keap1-Nrf2 signaling pathway

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ABSTRACT

Heat stress (**HS**) induces oxidative stress in the liver and affects health and production attributes in poultry birds. Bamboo leaf flavonoid (**BLF**) is a natural plant flavonoid that is effective in controlling oxidative stress. Therefore, the aim of current study was to investigate the impact of BLF on growth performance, liver index, serum biochemical parameters of liver function, liver antioxidant enzyme activities, and expression of genes and proteins related to the liver Keap1-Nrf2 system in cyclic heat stress (**CHS**) broilers. Twenty-eight-day old Arbor Acres broilers (n = 200) were randomly assigned to 5 groups. TN group fed basal diet was reared in a thermoneutral environment (24 ± 1 °C); CHS, 400 mg/kg BLF + CHS, 800 mg/kg BLF + CHS, and 1600 mg/kg BLF + CHS groups were reared in high temperature conditions (33 ± 1 °C, 8 h/day), with the basal diet supplemented with 0, 400, 800, and 1600 mg/kg BLF. The results indicated that ADG and ADFI of broilers in 28 to 35d and 36 to 42d CHS groups were significantly lower compared to the TN group. BLF improves growth performance of CHS broilers by increasing ADG, ADFI and decreasing F:G. BLF improved live weight, liver weight, liver index and reduced serum AST, ALP, ALT, T-BIL levels and increased TP levels in CHS broilers. Meanwhile, BLF supplementation enhanced the activity of hepatic antioxidant enzymes, resulting in higher T-AOC, CAT, GSH-PX and T-SOD levels than those of CHS broilers, and significantly reduced MDA levels. In addition, BLF downregulated the protein levels of Keap1 and P62, increased the expression levels of Nrf2 genes and proteins, and activated the expression of its downstream NQO1, HO-1, and SOD-1 antioxidant genes compared to CHS broilers. In summary, BLF regulates the expression of key genes and proteins in the Keap1-Nrf2 signaling pathway to alleviate liver injury in broilers by inhibiting oxidative stress, thereby promoting the growth performance of broilers with CHS.

Introduction

In recent years, chicken has become an indispensable source of meat in the routine diet of human beings due to its many advantages such as nutritional richness, low cost and high-quality protein (Liu et al., 2022). With increasing global warming and high-density feeding, heat stress (**HS**) negatively impacts the poultry industry by inhibiting growth performance, affecting meat quality, metabolism, redox state. Especially broilers, which are covered with feathers, have a high metabolism and lack sweat glands, are more susceptible to HS than mammals (Deng et al., 2023). It has been found that HS not only causes physiological and biochemical changes that reduce feed intake and growth in poultry, but also leads to varying degrees of liver injury and dysfunction by inducing mitochondrial stress and increasing reactive oxygen species (**ROS**) production (Emami et al., 2020). Oxidative stress is a major trigger for liver injury (Wan et al., 2024). Normally, broiler hepatocytes synthesize various antioxidant factor at a relatively constant rate (Bai et al., 2023), breaking down peroxides into harmless chemicals to protect the cells from oxidative stress. However, prolonged high-temperature environments can cause enhanced secretion of pro-oxidants and ROS, decrease the bioactivity of SOD, GPX, and T-AOC antioxidant molecules, and increase oxidative damage in broiler liver (Habashy et al., 2019). It is reported that natural compounds with antioxidant activity cannot only reduce the formation of ROS, which can effectively relieve oxidative stress and reduce HS negative impact to the poultry industry (Chobot et al., 2016).

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BLF is the main active ingredient in bamboo leaf extracts and is rich in flavonoids (Zhan et al., 2021). In recent years, BLF has received widespread attention in various fields including food production, pharmaceutical industry and livestock production due to its biological effects such as antioxidant, antibacterial, anticancer and antiviral effects (Nirmala et al., 2024). Dietary BLF was effective in increasing ADG and ADFI in broilers while reducing F:G and mortality (Cao et al., 2023). Flavonoids have a protective effect on the lipid redox process, and regulate the redox state of the body by enhancing the antioxidant capacity of broilers (Shen et al., 2019). Bamboo leaf extract showed protective effect against oxidative stress-induced liver injury by up-regulating the activity of antioxidant enzymes in serum and tissues of broilers and showed antioxidant defence by scavenging large number of free radicals in the body (Wu et al., 2024). Based on its chemical structure, BLF is able to act as a hydrogen donator to react with free radicals, exert excellent antioxidant ability by terminating free radical reactions, and inhibit ROS-induced macromolecular deterioration (Zhang et al., 2017). Previous studies have shown that flavonoids enhance antioxidant response element (**ARE**) binding activity and Nrf2 transcriptional activity and induce NQO2 and HO-1 expression (Sun et al., 2020). In addition, active substances flavonoids extracted from a variety of plants can modulate the Nrf2 antioxidant system and increase the expression of antioxidant factors, thereby alleviating oxidative damage caused by HS (Qin and Hou, 2017).

The liver is an important detoxification and metabolism organ and is more susceptible to HS damage than any other organ. HS exacerbates the load of ROS that the liver must neutralise, disrupting the body's buffer system and redox state and inducing oxidative stress (Salah et al., 2021). The Keap1-Nrf2 signaling pathway is an important defence mechanism for the body to inhibit oxidative damage (Chen et al., 2024). Under normal conditions, keap1 binds to Nrf2, which is subsequently degraded in the cytoplasm by ubiquitination. Under oxidative stress, the bridging protein Sqstm1/P62 induces Keap1-Nrf2 dissociation and promotes Nrf2 nuclear translocation. Nrf2 forms a heterodimer with Maf and activates ARE-dependent gene expression (Tang et al., 2022). In general, by increasing the transcription of Nrf2, oxidative stress and lipid peroxidation can be effectively inhibited, thereby preventing and reducing liver injury (Zhou et al., 2022). However, there are fewer studies on whether BLF can alleviate liver injury in CHS-exposed broilers by modulating the Keap1-Nrf2 system. Therefore, the aim of this study was to investigate the mitigating effect of exogenous BLF on oxidative damage in the liver of broiler chickens exposed to CHS and the potential protective mechanisms.

Materials and methods

Location and ethics statement

The broiler feeding trials were completed in the animal testing area of Anhui Science and Technology University. Sample analyses were carried out in the Anhui Province Key Laboratory of Animal Nutrition Regulation and Health. The ethical approval was obtained from the Institutional Animal Care and Use Committee of Anhui Science and Technology University, China (Approval No. 2022002).

Animal and experimental design

Two hundred healthy Arbor Acres broilers (aged 1d, equal males and females) were sourced from a local farm (Fengyang, China) and reared for 1 to 27 days in a commercial standard brooding pattern. Subsequently, broilers were freely assigned to 5 experimental groups of 4 replicate pens of 10 broilers each for 14 days. The first group of broilers were raised in a thermo-neutral environment (TN; 24 ± 1 °C) and fed on a basal diet. Other experimental groups of broilers were exposed to high temperature conditions for 8 h/day (33 ± 1 °C; from 09:00 to 17:00 h) and fed on a basal diet of 0 (CHS), 400 (400mg/kg BLF + CHS), 800 (800mg/kg BLF + CHS), and 1600 (1600mg/kg BLF + CHS) mg/kg BLF. Broilers of 28 - 42 d were used in this experiment because it was considered that the sensitivity to CHS was higher in this growth stage than in chicks. The poultry farm monitoring system (ROTEM AC-2000 PLUS, AgroLogic Ltd, Israel) was used to control the temperature and relative humidity in chicken houses. The extra heat for CHS groups was provided by an industrial heater (JiYi: IFH04-30 A), heat up at 9:00 a.m. and turn off to cool down at 5:00 p.m. (the temperature of the coop could be raised to about 33 °C and lowered to about 24°C in 2–3 h in natural state), and adjust the temperature and humidity in time according to the condition of the chicken coop. BLF was obtained from Shaanxi Baichuan Biotechnology Co., Ltd (Shaanxi, China), with a total flavonoid content of > 90 %, while the rest of the components consisted of polyphenols, amino acids, polysaccharides, and alkaloids. The relative ambient humidity of the coop was maintained at 60 % ± 5 % during our experiments. The relative ambient humidity of the the coop was maintained at 60 % ± 5 %. Broilers from each replicate pen were housed in wire cages with dimensions of 100 (length) × 80 (width) × 60 (height) cm, with free availability of feed and water. During our experiment, we recorded body weights and the amount of feed consumed per day, and then analyzed average daily feed intake (**ADFI**), average daily weight gain (**ADG**), and feed to weight ratio (**F:G**). Basic diet and nutrient composition (Table 1) is according to National Research Council: Nutritional Requirements of Poultry.

Table 1
Composition of the basal diet at two time periods during the experiment (%).

Feed Ingredients	1-21d	22-42d
Maize	60.90	62.90
Soybean pulp	28.10	26.80
Soybean oil	3.50	3.50
Fish powder	3.50	3.50
$CaHPO_4$	1.50	1.50
NaCl	0.50	0.50
Premix[1]	2.00	2.00
Total	100.00	100.00
Nutrient levels[2]		
ME/(MJ/kg)	12.86	13.04
Crude protein (%)	22.60	22.01
Crude fat	5.88	5.96
Crude fiber	2.49	2.44
Crude ash	3.65	3.60
Lys (%)	1.06	1.02
Ca (%)	1.20	1.20
P (%)	0.70	0.67

[1] The premix provided per kilogram of diets: manganese ($MnSO_4$), 66 mg; zinc (ZnO), 44 mg; copper ($CuSO_4$), 9 mg; iron ($FeSO_4$) 50 mg; I (KI), 0.4 mg; vitamin A, 7, 000 IU; vitamin D_3, 875 IU; vitamin E, 20 IU; vitamin K_3, 1 mg; vitamin B_2, 4.5 mg; vitamin B_6, 2.5 mg; vitamin B_{12}, 0.6 mg; nicotinic, 50 mg; D-pantothenic, 12 mg.
[2] ME was a calculated value.

Sample collection

At 7 and 14 d of CHS, eight overnight fasted broilers were chose from each experimental group and weighed. Blood samples were taken from the jugular vein into an anticoagulant-free vacuum tube and centrifuged for 20 min (3000 rpm/min; 4°C).

Serum was dispensed and kept at −20 °C for serum biochemical analyses of liver function. Broilers were euthanized by bloodletting through the jugular vein. Livers were stripped and weighed to assess the liver index: liver index = liver weight/body weight × 100 %. Liver tissue samples are then dispensed and placed in freezing tubes, where they are put into liquid nitrogen and immediately frozen at −80 °C for storage.

Serum biochemical analysis of liver function

Serum liver function indices was measured by a fully automated

Table 2
Target gene primer sequence for RT-qPCR.

Gene[1]	Primer sequence (5′–3′)	PCR product size, bp	Accession no.
Nrf2	F-CTGCTAGTGGATGGCGAGAC R-CTCCGAGTTCTCCCCGAAAG	132	NM_001030756.1
HO-1	F-AAACTTCGCAGCCACACAAC R-GACCAGCTTGAACTCGTGGA	155	NM_205344.2
NQO1	F-CCCGAGTGCTTTGTCTACGAGATG R-ATCAGGTCAGCCGCTTCAATCTTC	107	NM_001277620.2
SOD-1	F-CGCTCGTAGGTGGTTGT R-GCTGCTGGAAGTGGATG	124	NM_205064.2
GPX1	F-CTGCAACCAATTCGGGCAC R-CGCACTTCTCGAACATGGTG	116	NM_001277853.3
β-actin	F-CCGCTCTATGAAGGCTACGC R-CTCTCGGCTGTGGTGGTGAA	128	NM_205518.2

[1] *F* = forward; *R* = reverse; Nrf2 = Nuclear factor erythroid 2-related factor 2; NQO1 = NAD (P)H/quinone oxidoreductase 1; HO-1 = heme oxygenase-1; SOD-1 = superoxide dismutase; GPX-1 = glutathione peroxidase.

biochemical analyser (BS-200, Mindray, Shenzhen, China) and an accompanying kit. These include aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), alkaline phosphatase (**ALP**), total protein (**TP**), albumin (**ALB**), total bilirubin (**T-BIL**), and direct bilirubin (**D-BIL**).

Antioxidant enzyme activity analysis

Total antioxidant capacity (**T-AOC**; no. A015-2-1), total superoxide dismutase (**T-SOD**; no. A001-3-2), glutathione peroxidase (**GSH-PX**; no. A005-1-2) and catalase (**CAT**; no. A007-1-1) activities and malondialdehyde (**MDA**; no. A003-1-2) concentrations of the hepatic tissues were determined by the kit obtained by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Quantitative real-time pCR

Liver samples were homogenised in RNAiso Plus reagent (Takara, Shiga, Japan, 9108) and total RNA was extracted. RNA quality was assessed using a NanoDrop One (Thermo Scientific, Wilmington, MA, USA). cDNA was synthesised from the extracted RNA using the Prime Script RT Master kit (Takara, Shiga, Japan, RR047A). PCR reaction solutions were prepared based on the reaction system of the TB GreenTM Premix Ex TaqTM II kit (Tokyo, Japan). The target genes were then amplified using the Roche LightCycler 480 System (Roche, Switzerland). The gene sequences of the antioxidant-related factors studied are presented in Table 2, where β-actin was served as a housekeeping gene. Data were processed using the $2^{-\Delta\Delta Ct}$ method to assess the expression levels of the goal genes.

Western blotting

Total protein was extracted from liver tissue using lysis buffer containing Refrigerato RIPA and phenylmethylsulfonyl fluoride enzyme inhibitor (**PMSF**). Protein concentration was determined using a BCA kit (No. P0010S, Beyotime, Shanghai, China) and adjusted to the same concentration with lysis buffer. Subsequently, the quantified proteins and 5 × loading buffer (No. P0015L, Beyotime, Shanghai, China) were mixed proportionally and boiled at 95 °C for 10 min. 20μg of sample was separated by electrophoresis at 80 V in a 10 % SDS-PAGE gel and then transferred to a polyvinylidene difluoride (**PVDF**) membrane for 90 min. Closed with 5 % skimmed milk powder for 2 h and incubated with Keap1, Nrf2, Sqstm1/P62 and β-actin antibodies at 4°C overnight. After incubating the membrane with rabbit/mouse IgG secondary antibody, protein bands were visualised using an ECL kit (Beyotime, China) and a chemiluminescence imaging system. The grey level of the protein bands was assessed using ImageJ software.

Statistic analysis

In this study, SPSS 27.0 (SPSS Inc., Chicago, USA) system was adopted for statistical and data processing. Values are expressed as mean ± standard error. Differences were compared by one-way analysis of variance (**ANONA**), and a statistically significant difference existed when $P < 0.05$.

Results

BLF improves the growth performance in CHS broilers

The growth performance of broilers is usually evaluated in terms of ADFI, ADG and F:G (Fig. 1) . Relative to TN group, ADFI and ADG were significantly reduced ($P < 0.05$) in the CHS group at 28-35d and 36-42d, and supplementation with BLF had a remarkable improvement ($P < 0.05$). F:G at 28 to 35d 800mg/kg BLF and 36 to 42d 400mg/kg BLF was remarkably reduced ($P < 0.05$) relative to the CHS group.

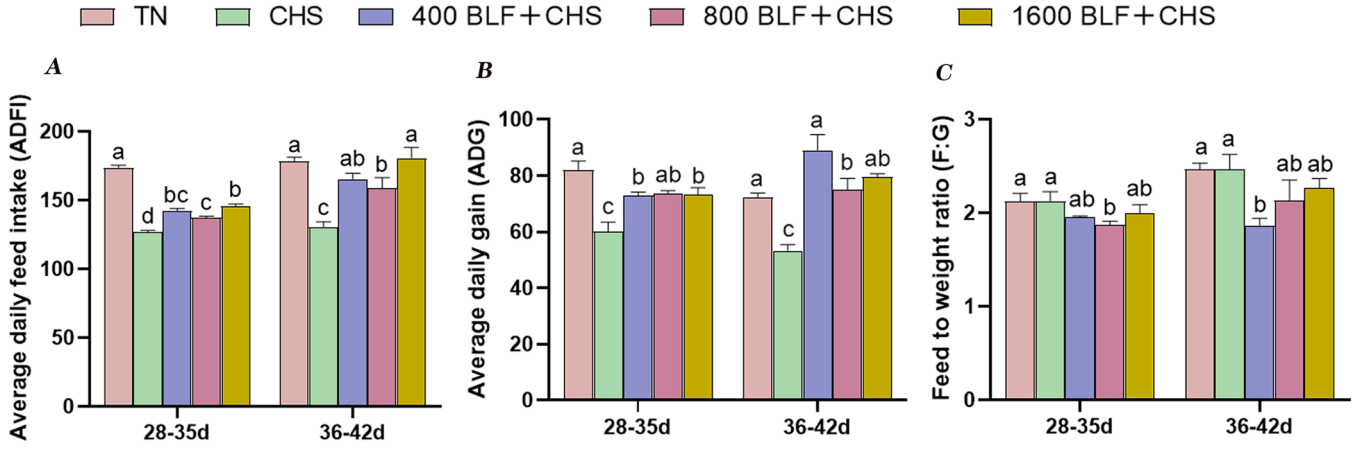

Fig. 1. Impact of BLF on ADFI, ADG, and F:G in CHS broilers. TN, thermoneutral temperature (24 ± 1 °C) + basal diet; CHS, 400 BLF + CHS, 800 BLF + CHS, 1600 BLF + CHS, high-temperature environments (33 ± 1 °C, 8 h/day and from 09:00 to 17:00 h) + 0, 400, 800, and 1600 mg/kg BLF of the basal diet. A, average daily feed intake (**ADFI**); B, average daily weight gain (**ADG**); C, feed to weight ratio (F:G). Values are SD ± mean. Different lowercase letters represent significant differences $P < 0.05$.

Table 3
Effect of BLF on live weight, liver weight and liver index in CHS broilers.

Age (days)[1]	Items[2]	TN	CHS	400mg/kg BLF+CHS	800mg/kg BLF+CHS	1600mg/kg BLF+CHS
35d	Body weight (kg)	2.25 ± 0.13^{a}	2.02 ± 0.18^{b}	2.03 ± 0.09^{b}	1.99 ± 0.13^{b}	1.98 ± 0.14^{b}
	Liver weight (g)	38.24 ± 2.27^{a}	34.76 ± 2.9^{b}	35.03 ± 1.88^{b}	35.52 ± 1.06^{b}	34.99 ± 1.42^{b}
	Liver index (%)[3]	1.70 ± 0.08	1.72 ± 0.07	1.73 ± 0.12	1.78 ± 0.09	1.77 ± 0.08
42d	Body weight (kg)	2.54 ± 0.08^{a}	2.26 ± 0.16^{b}	2.67 ± 0.15^{a}	2.53 ± 0.11^{a}	2.53 ± 0.09^{a}
	Liver weight (g)	43.90 ± 2.45^{a}	35.05 ± 2.60^{c}	42.36 ± 2.01^{a}	42.74 ± 1.16^{a}	39.48 ± 1.60^{b}
	Liver index (%)	1.73 ± 0.12^{a}	1.55 ± 0.13^{c}	1.59 ± 0.09^{bc}	1.69 ± 0.04^{ab}	1.56 ± 0.06^{c}

[1] TN, thermoneutral temperature (24 ± 1°C) + basal diet; CHS, 400 mg/kg BLF + CHS, 800 mg/kg BLF + CHS, 1600 mg/kg BLF + CHS, high-temperature environments (33 ± 1°C, 8 h/day and from 09:00 to 17:00 h) + 0, 400, 800, and 1600 mg/kg BLF of the basal diet.
[2] Results are expressed as mean ± SD. a-d with different superscript letters in the same column indicate remarkable differences ($P < 0.05$).
[3] liver index = liver weight/body weight × 100 %.

Effect of BLF on liver index in CHS broilers

As displayed in Table 3, Relative to the TN group, broiler weights and liver weights were remarkably lower ($P < 0.05$) in the CHS group at 35d and 42d, and liver index was remarkably lower ($P < 0.05$) at 42d. Compared with the CHS group, broiler weight and liver weight were elevated ($P < 0.05$) in the BLF-supplemented group at 42d, and liver index was remarkably elevated ($P < 0.05$) in broilers adding with 800 mg/kg BLF.

Effect of BLF on serum liver function indexes of CHS broilers

We evaluated a number of serum biochemical parameters responsive to liver injury see Tables 4 and 5. Relative to the TN group, CHS broilers had higher levels of serum AST, ALP, and T-BIL ($P < 0.05$) and lower levels of TP ($P < 0.05$) at 35d, and higher levels of serum AST and ALT ($P < 0.05$) and lower levels of TP ($P < 0.05$) at 42d. Relative with the CHS group, BLF remarkably reduced ($P < 0.05$) AST (400 mg/kg BLF), ALP (800, 1600 mg/kg BLF), and T-BIL (800 mg/kg BLF) levels at 35d, and AST (400, 800, 1600 mg/kg BLF), and ALT (800 mg/kg BLF) levels at 42d. Also significantly elevated ($P < 0.05$) TP (400 mg/kg BLF) levels.

BLF alleviates CHS induced oxidative damage in broiler liver

Indicators of antioxidant status of broiler liver are shown in Tables 6 and 7. Compared to the TN group, CHS remarkably elevated ($P < 0.05$) the MDA levels in the liver. Meanwhile, it was detected that CHS group induced lower ($P < 0.05$) levels of T-AOC and CAT at 35d as well as T-AOC, GSH-PX, and T-SOD at 42d. Dietary BLF supplementation demonstrated a palliative effect compared to the CHS group. BLF

Table 6
Effect of BLF on redox status of CHS broiler liver in 35d.

Items[1]	TN	CHS	400mg/kg BLF+CHS	800mg/kg BLF+CHS	1600mg/kg BLF+CHS
T-AOC (U/mg prot)	97.18 ± 5.69^{a}	87.92 ± 8.58^{b}	90.54 ± 7.13^{ab}	95.77 ± 4.54^{a}	80.26 ± 4.14^{c}
CAT (U/mg prot)	12.89 ± 0.93^{b}	11.30 ± 0.62^{c}	13.92 ± 1.13^{ab}	14.30 ± 0.92^{a}	14.17 ± 1.16^{a}
GSH-PX (U/mg prot)	31.11 ± 2.40	30.76 ± 1.81	31.13 ± 2.86	28.77 ± 2.63	28.62 ± 1.51
T-SOD (U/mg prot)	90.09 ± 8.47^{ab}	84.93 ± 6.80^{b}	96.72 ± 11.95^{ab}	97.65 ± 11.11^{a}	89.71 ± 11.18^{ab}
MDA (nmol/mg prot)	0.40 ± 0.09^{d}	0.69 ± 0.16^{a}	0.47 ± 0.11^{cd}	0.55 ± 0.07^{bc}	0.63 ± 0.12^{ab}

Table 4
Effect of BLF on serum liver function indices in broilers at 35d.

Items[1]	TN	CHS	400mg/kg BLF+CHS	800mg/kg BLF+CHS	1600mg/kg BLF+CHS
AST (U/L)	259.39 ± 34.76^{b}	395.15 ± 73.05^{a}	308.05 ± 44.6^{b}	321.27 ± 60.51^{ab}	309.40 ± 63.26^{ab}
ALT (U/L)	14.96 ± 3.83	15.40 ± 3.28	13.77 ± 1.15	13.39 ± 2.21	13.89 ± 1.71
ALP (U/L)	965.35 ± 187.91^{c}	1872.13 ± 277.87^{a}	1554.72 ± 435.88^{ab}	1193.40 ± 376.58^{bc}	1318.15 ± 174.36^{bc}
TP (g/L)	30.89 ± 1.25^{a}	27.36 ± 1.25^{b}	31.51 ± 2.85^{a}	28.04 ± 1.79^{b}	29.80 ± 2.51^{ab}
ALB (g/L)	13.18 ± 0.64	12.10 ± 0.68	13.17 ± 0.81	12.00 ± 0.93	11.68 ± 0.81
T-BIL(μmol/L)	7.21 ± 0.79^{c}	8.51 ± 0.79^{a}	8.15 ± 0.69^{ab}	7.45 ± 0.82^{bc}	7.61 ± 0.72^{abc}
D-BIL(μmol/L)	6.85 ± 0.71	6.59 ± 0.93	6.98 ± 0.80	7.16 ± 0.62	6.88 ± 0.77

Table 5
Effect of BLF on serum liver function indices in broilers at 42d.

Items[1]	TN	CHS	400mg/kg BLF+CHS	800mg/kg BLF+CHS	1600mg/kg BLF+CHS
AST (U/L)	349.49 ± 25.50^{b}	469.32 ± 34.51^{a}	369.75 ± 48.59^{b}	273.47 ± 40.86^{c}	377.20 ± 18.17^{b}
ALT (U/L)	13.39 ± 1.27^{b}	18.28 ± 2.47^{a}	14.80 ± 2.96^{ab}	13.55 ± 3.14^{b}	18.05 ± 1.70^{a}
ALP (U/L)	1253.63 ± 144.47	1572.23 ± 210.02	1364.90 ± 423.63	1219.63 ± 345.77	1382.95 ± 397.44
TP (g/L)	31.02 ± 3.87^{a}	23.58 ± 4.96^{c}	29.47 ± 1.95^{ab}	23.46 ± 3.88^{c}	26.93 ± 2.18^{bc}
ALB (g/L)	11.62 ± 0.91	11.02 ± 0.82	11.92 ± 0.98	11.23 ± 0.48	11.70 ± 1.21
T-BIL(μmol/L)	7.86 ± 0.61	8.20 ± 0.83	8.11 ± 0.87	7.65 ± 0.87	8.11 ± 0.66
D-BIL(μmol/L)	5.53 ± 0.79	5.63 ± 0.62	5.57 ± 0.85	5.64 ± 0.60	5.99 ± 0.66

[1] AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; TP = total protein; ALB = albumin; T-BIL = total bilirubin; D-BIL = direct bilirubin. TN, thermoneutral temperature (24 ± 1°C) + basal diet; CHS, 400 mg/kg BLF + CHS, 800 mg/kg BLF + CHS, 1600 mg/kg BLF + CHS, high-temperature environments (33 ± 1°C, 8 h/day and from 09:00 to 17:00 h) + 0, 400, 800, and 1600 mg/kg BLF of the basal diet. Results are expressed as mean ± SD. a-d with different superscript letters in the same column indicate remarkable differences ($P < 0.05$).

Table 7
Effect of BLF on redox status of CHS broiler liver in 42d.

Items[1]	TN	CHS	400mg/kg BLF+CHS	800mg/kg BLF+CHS	1600mg/kg BLF+CHS
T-AOC (U/mg prot)	82.03 ± 14.72^{a}	59.90 ± 12.95^{b}	75.26 ± 9.45^{a}	80.93 ± 11.35^{a}	78.65 ± 11.38^{a}
CAT (U/mg prot)	10.43 ± 1.47	12.26 ± 1.11	11.80 ± 2.61	12.88 ± 2.81	11.46 ± 1.93
GSH-PX (U/mg prot)	25.12 ± 3.60^{a}	20.06 ± 1.98^{b}	21.95 ± 3.12^{ab}	25.30 ± 3.43^{a}	20.38 ± 4.52^{b}
T-SOD (U/mg prot)	99.63 ± 11.55^{a}	75.44 ± 14.52^{c}	81.91 ± 6.84^{bc}	88.34 ± 15.83^{ab}	78.17 ± 8.27^{bc}
MDA (nmol/mg prot)	0.38 ± 0.10^{c}	0.67 ± 0.20^{a}	0.64 ± 0.26^{a}	0.61 ± 0.14^{ab}	0.47 ± 0.06^{bc}

[1] T-AOC = total antioxidant capacity; T-SOD = total superoxide dismutase; GSH-PX = glutathione peroxidase; CAT = catalase; MDA = malondialdehyde. TN, thermoneutral temperature (24 ± 1°C) + basal diet; CHS, 400 mg/kg BLF + CHS, 800 mg/kg BLF + CHS, 1600 mg/kg BLF + CHS, high-temperature environments (33 ± 1°C, 8 h/day and from 09:00 to 17:00 h) + 0, 400, 800, and 1600 mg/kg BLF of the basal diet. Results are expressed as mean ± SD. a-d with different superscript letters in the same column indicate remarkable differences ($P < 0.05$).

elevated ($P < 0.05$) T-AOC (800 mg/kg BLF), CAT (400, 800, 1600 mg/kg BLF), and SOD (800 mg/kg BLF) levels at 35d, and T-AOC (400, 800, 1600 mg/kg BLF), GSH-PX (800 mg/kg BLF), and T-SOD (800 mg/kg BLF) levels at 42d ($P < 0.05$). In addition, BLF significantly reduced ($P < 0.05$) liver MDA content in CHS broilers.

BLF activates gene expression related to the Nrf2 pathway in CHS broiler liver

We assessed the expression levels of Nrf2 as well as its downstream antioxidant genes using quantitative real-time PCR as shown in Fig. 2. Compared to the TN group, CHS reduced ($P < 0.05$) the gene expression of Nrf2, NQO1, and HO-1 at 35d and Nrf2, HO-1, and SOD-1 at 42d. Dietary addition of BLF elevated the expression levels of Nrf2 related genes in CHS broiler livers. Compared to the CHS group, the expression levels of Nrf2 (400, 800 mg/kg BLF), NQO1 (400, 800, 1600 mg/kg BLF), and HO-1 (800, 1600 mg/kg BLF) genes were significantly elevated ($P < 0.05$) in the BLF group at 35d; The expression levels of Nrf2 (800 and 1600 mg/kg BLF), SOD-1 (400, 800 mg/kg BLF), and HO-1 (800, 1600 mg/kg BLF) genes were elevated ($P < 0.05$) in the BLF group at 42d.

BLF activates protein expression associated with Keap1-Nrf2 signaling pathway in CHS broiler liver

We assessed the protein levels of Keap1, Nrf2, and P62 by Western blot (Fig. 3). Compared with the TN group, CHS enhanced ($P < 0.05$) Keap1 and P62 protein expression and reduced ($P < 0.05$) Nrf2 protein expression at 35d. CHS downregulated ($P < 0.05$) Nrf2 protein levels at 42d. Relative to the CHS group, 800 mg/kg BLF up-regulated ($P < 0.05$) the liver Nrf2 protein expression in broilers, while decreasing ($P < 0.05$) Keap1 (800, 1600 mg/kg BLF) and P62 (400, 800, 1600 mg/kg BLF) protein levels at 35d.

Discussion

In animal feeding, poultry are subject to a range of non-specific defense responses to sustained high ambient temperatures, including elevated body temperature, reduced appetite and suppressed production

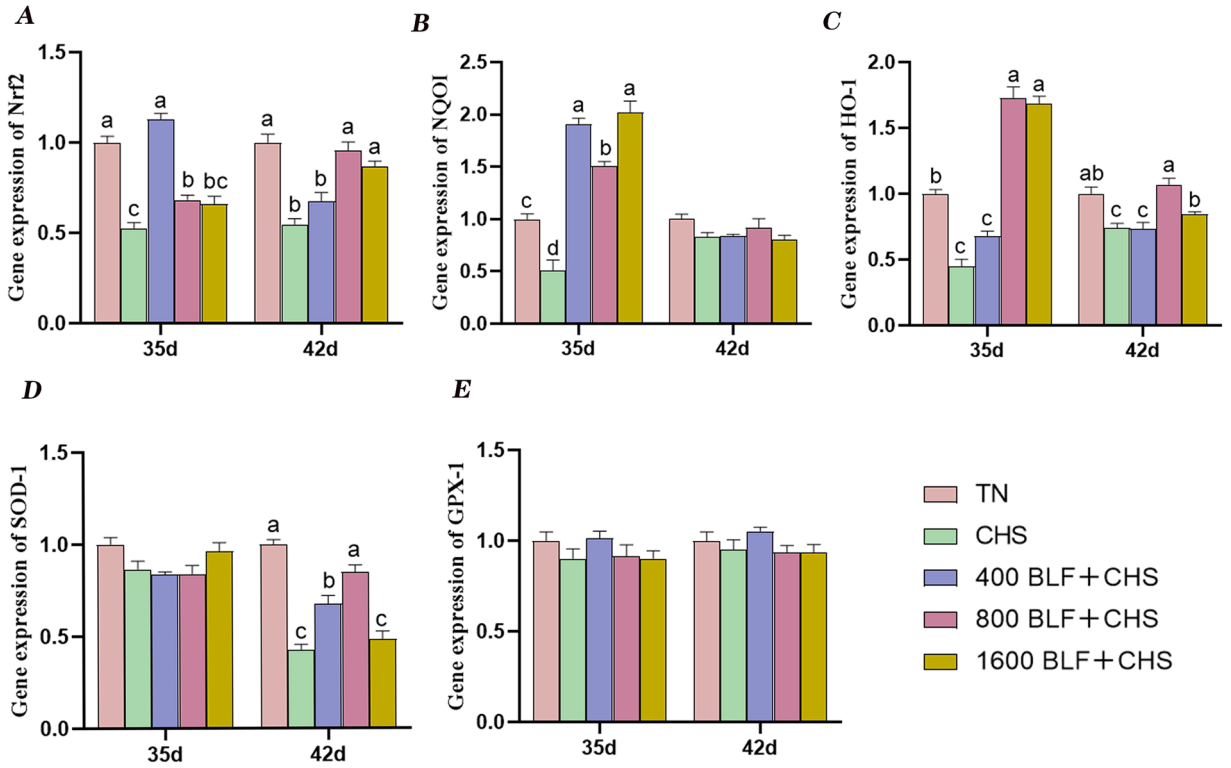

Fig. 2. Effects of BLF on the gene expressions of (A) Nrf2, (B) NQO1, (C) HO-1, (D) SOD-1, (E) GPX-1 in the liver of CHS broilers. Nrf2 = Nuclear factor erythroid 2-related factor 2; NQO1 = NAD (P)H/quinone oxidoreductase 1; HO-1 = heme oxygenase-1; SOD-1 = superoxide dismutase; GPX-1 = glutathione peroxidase. Values are SD ± mean. Different lowercase letters represent significant differences $P < 0.05$.

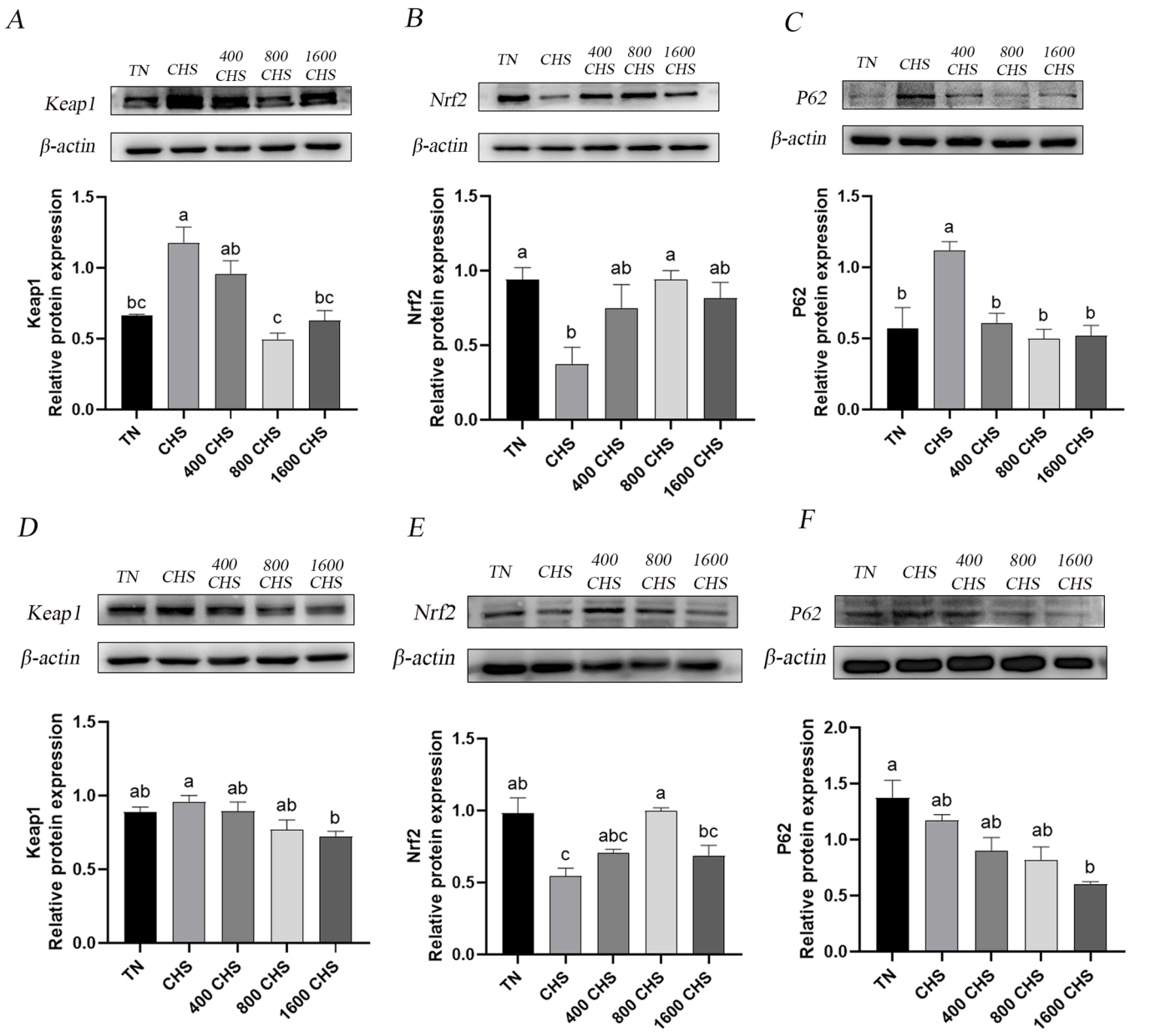

Fig. 3. Effect of BLF on the expression of Keap1 (A, D), Nrf2 (B, E), and p62 (C, F) proteins in the liver of CHS broilers at 35d (A, B, C) and 42d (D, E, F). TN, thermoneutral temperature (24 ± 1°C) + basal diet; CHS, 400 CHS, 800 CHS, 1600 CHS, high-temperature environments (33 ± 1°C, 8 h/day and from 09:00 to 17:00 h) + 0, 400, 800, and 1600 mg/kg BLF of the basal diet. Keap1 = kelch-like ECH associated protein 1; Nrf2 = nuclear factor erythroid 2-related factor 2; p62 = sequestosome 1. Values are SD ± mean. Different lowercase letters represent significant differences $P < 0.05$.

performance (Li et al., 2024). High ambient temperatures cause decreased feed intake in broilers during mid to late life and negatively affect growth and development by disrupting energy metabolism and inducing oxidative stress in the body (Tang et al., 2022). In addition, HS activated the hypothalamic-pituitary-adrenal axis, down-regulated the sympathetic excitability of the feeding center and the digestive tract, and significantly reduced ADFI and ADG in broilers (Brugaletta et al., 2021). We obtained similar results (Lu et al., 2023), that is CHS significantly reduced ADG and ADFI in broilers and further found that supplementation of BLF effectively improved ADG, ADFI and decreased F:G. The main active components of BLF are flavonoids, in addition to polyphenols, amino acids, and polysaccharide substances, which can be used as growth hormone to enhance growth performance in livestock (Chen et al., 2016). It's reported that the addition of BLF to Arbor Acres broiler diets counteracted the growth inhibitory effects of HS in broilers by increasing ADFI and body weight (Shen et al., 2019). In addition, BLF has a specific flavor that improves the palatability of feed and increases feed intake. These results suggest that exogenous BLF can enhance the growth performance of CHS broilers.

Serum liver function parameters reveal the extent of host liver injury, such as AST, ALT and T-BIL, and their increased levels indicate hepatocellular deterioration (Ahmed et al., 2021). Organ weight is an important parameter in understanding the developmental status of an organ. In the present study, CHS increased broiler serum ALT, ALP, AST and T-BIL activities and decreased TP levels. At the same time, we detected lower relative weight and fresh weight parameters of CHS broiler livers. This means that CHS broilers may have liver dysfunction. CHS induces excessive accumulation of reactive ROS in broiler liver and causes oxidative stress, which is the main reason for liver damage (Luo et al., 2019). Adding natural antioxidants to livestock diets is an effective way to improve CHS-induced liver dysfunction (Soto-Alarcon et al., 2019). Flavonoids efficiently inhibit the expansion of hepatic stellate

cells and collagen production, enhance the differentiation and reproduction of hepatocytes, and play a role in liver protection. In addition, exogenous BLF supplementation induced a decrease in the biochemical levels of several indicators of liver dysfunction in broilers, namely AST, ALT, and T-BIL (Cao et al., 2022), and their reduced concentrations effectively mitigated liver injury in CHS broilers. Our study is consistent with the above findings that BLF alleviates liver dysfunction in broilers exposed to CHS.

ROS generated by oxidative stress can disrupt the redox balance and cause damage to all biomolecules such as DNA, proteins and fats, thus negatively affecting the growth and development of livestock and poultry (Kishawy et al., 2023). When oxidative stress occurs, the body's enzymatic antioxidant systems play a primary role, such as SOD, GSH-PX, and CAT, which work synergistically to disperse excess free radicals. MDA is the end product of lipid peroxidation and reflects the extent of oxidative stress and free radical reactions. HS causes mitochondrial stress in broiler livers, and dysregulation of the body's ROS removal process results in the production of excess ROS, which rapidly depletes the body's antioxidants (Jing et al., 2023; Yang et al., 2020). In the present study, it was found that oxidative stress may be present in these CHS birds at 33°C due to significant elevated liver MDA as well as a significant reduction in antioxidant enzyme activities in CHS broilers. It is worth noting that we found that as CHS persisted, the antioxidant enzyme activity in the liver of 42d broilers was lower than that of 35d. During heat stress, the mitochondria in broilers are responsible for the production of superoxide anions as a response to oxidative stress in order to neutralize the harmful effect of the increased levels of free radicals (Egbuniwe et al., 2018). The persistence of CHS further inhibits the conversion of ROS to water and O_2 by enzymatic and non-enzymatic antioxidants, resulting in a progressive decrease in antioxidant capacity. However, we found that BLF supplementation suppressed the increase in liver MDA levels in CHS broilers, implying that BLF treatment alleviated CHS induced oxidative stress. In addition, we found that BLF alleviated oxidative stress in CHS broiler liver by elevating the activity of antioxidant enzymes. Bioflavonoids regulate the antioxidant defense system in the body, with 3 to 4 hydroxyl groups present in their chemical structure, which have the ability to block and eliminate free radicals and avoid the formation of oxidative damage. It can be hypothesised that the alleviation of oxidative stress in CHS broiler livers by BLF that we observed may be due to the antioxidant properties of BLF.

Nrf2 is a redox-sensitive nuclear transcription factor that regulates oxidative damage in the organism by combining AREs present in the nucleus (Shaw and Chattopadhyay, 2020). Oxidative stress is a major factor detrimental to broiler growth and development and is an important cause of liver dysfunction (Liu et al., 2020). Prolonged high-temperature conditions lead to rapid depletion of Nrf2 in the organism and reduce its protein and gene level as well as the level of downstream antioxidant genes. Cao et al. (2023) found that HS reduced the level of Nrf2 mRNA in broiler liver and inhibited the gene levels of its downstream antioxidants SOD, GST, and HO-1, which disrupted the balance of hepatic redox system and led to relatively slow growth of broilers. In the present study, CHS significantly reduced the gene levels of Nrf2, HO-1 and SOD-1 in broiler liver, again confirming the presence of oxidative stress in broiler liver under CHS conditions. We found that the reduced growth performance could also be attributed to CHS-induced oxidative stress in broiler liver. Dietary BLF supplements usually increase Nrf2 as well as its related gene expression. Previous studies have shown that BLF activates antioxidant defence responses against oxidative stress and prevents oxidative damage *in vivo* by increasing Nrf2/HO-1/NQO1 gene levels (Yu et al., 2019). Under CHS conditions, exogenous flavonoids further blocked the ubiquitination and degradation of Nrf2 and promoted its nuclear translocation, leading to increased expression of Nrf2 and its downstream antioxidant genes to ameliorate HS injury in broilers. Our experiments yielded similar conclusions that exogenous BLF supplementation significantly upregulated the mRNA expression levels of Nrf2 and HO-1 in broiler liver. BLF

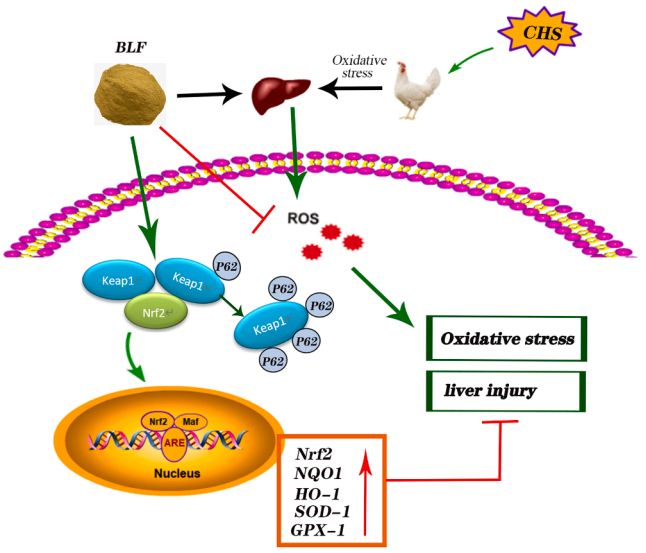

Fig. 4. The schematic model illustrates the mechanism by which BLF alleviates CHS-induced oxidative stress and liver injury in broiler liver. ROS = reactive oxygen species; Keap1 = kelch-like ECH associated protein 1; Nrf2 = nuclear factor erythroid 2-related factor 2; p62 = sequestosome 1; NQO1 = NAD (P)H/quinone oxidoreductase 1; HO-1 = heme oxygenase-1; SOD-1 = superoxide dismutase; GPX-1 = glutathione peroxidase; ARE = antioxidant response element.

regulates oxidative stress in broiler liver by activating Nrf2, thereby reducing CHS induced liver injury.

We further used western blotting to test how CHS causes liver injury and whether BLF can activate the Keap1-Nrf2 system to alleviate liver oxidative stress caused by CHS. The Keap1-Nrf2 system, the body's primary defense mechanism against environmental injury, is a classic two-component system: Keap1, a cysteine-thiol-rich sensor of redox injury, and Nrf2, a synergistic effector that exerts protective effects. P62 is a substrate that needs to be degraded by autophagic digestion, and an excess of P62 induces a large amount of ROS production, causing DNA damage, oxidative stress, and so on. Earlier studies found (Ding et al., 2023) that HS inhibited the activation of the Nrf2-Keap1 pathway in broiler liver by decreasing the protein expression of Nrf2 and HO-1 and enhancing the protein expression of Keap1 and P62, which is similar to our results. In this study results showed that CHS increased protein levels of Keap1, P62 and inhibited Nrf2 protein expression in broiler liver. Subsequently, we found that dietary BLF alleviated the inhibitory effect of CHS on the Nrf2-Keap1 system by increasing Nrf2 protein expression and decreasing the protein level of Keap1. High intensity CHS, which disrupts the redox balance system of the organism, causes liver dysfunction, which greatly affects Nrf2 protein formation and thus reduces broiler growth performance by inhibiting the Keap1-Nrf2 pathway in the broiler liver. BLF can act as an activator of the Keap1-Nrf2 system, decreasing Keap1 protein levels, and the generated Nrf2 can be directly translocated into the nucleus and form dimers with Maf proteins. The oxidative damage was then mitigated by recognizing ARE and enhancing the gene levels of SOD-1 and HO-1 (Zhang et al., 2017). These results further reinforce the beneficial effects of BLF on liver injury in CHS broilers. Therefore, it is reasonable to believe that dietary BLF can reverse hepatic oxidative stress in CHS broilers by modulating the Keap1-Nrf2 pathway, thereby improving growth performance.

Conclusions

The present study revealed that CHS reduced hepatic antioxidant enzyme activities and inhibited the activation of the Keap1-Nrf2 signaling pathway in broiler chickens, resulting in hepatic oxidative

stress, which induced a decline in broiler growth performance. Dietary BLF supplementation reduces protein levels of hepatic Keap1 and P62, which contributes to increased gene and protein expression of Nrf2, which translocates to the nucleus to activate its downstream levels of related antioxidant genes. Increased expression of these key antioxidant genes synergistically improves antioxidant capacity and alleviates liver oxidative stress and liver injury caused by CHS (Fig. 4). Meanwhile, BLF promoted liver development, increased antioxidant enzyme activities and improved serum biochemical liver function indexes in CHS broilers. To sum up, dietary BLF supplementation can alleviate CHS-induced liver injury and growth inhibition in broilers. These findings provide new insights into the use of BLF as a novel antioxidant for mitigating against oxidative damage in CHS broilers.

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that may have influenced the work reported in this study.

Acknowledgments

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