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Lactobacillus reuteri alleviates diquat induced hepatic impairment and mitochondrial dysfunction via activation of the Nrf2 antioxidant system and suppression of NF-κB inflammatory response

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ABSTRACT

Accumulating evidence has shown that elevated oxidative stress and inflammatory response leads to hepatic impairment and dysfunction of hens during the aging process. This study was conducted to investigate the potential regulatory mechanisms of *Lactobacillus reuteri* (*L. reuteri*) in alleviating hepatic oxidative stress and dysfunction induced by diquat (DQ) exposure. A total of 480 48-wk-old Jingbai hens were randomly assigned to 4 groups: control group (Con), *L. reuteri* group (L.R), diquat-challenged group (DQ), and *L. reuteri* protective group (L.R+DQ). The results demonstrated that DQ exposure induced oxidative damages and lipid metabolism disorders manifested as the elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, triglyceride (TC) contents in serum and lipid accumulation in liver. *L. reuteri* supplementation alleviated DQ-induced liver oxidative injury, reflected by repairing the morphology of liver and decreasing the AST and ALT activities in serum. *L. reuteri* decreased the hepatic malonaldehyde (MDA) accumulation and enhanced the total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) activities in liver through regulating the nuclear factor erythroid 2-related factor 2 (Nrf2) and hemeoxygenase-1 (HO-1) mediated antioxidant system. In addition, *L. reuteri* curtailed reactive oxygen species (ROS) production and mitigated the depletion of membrane potential and thus recovering mitochondrial function disturbed by DQ challenge. Moreover, *L. reuteri* inhibited hepatic toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)/nuclear factor-kappa B (NF-κB) pathway activation, downregulated the pro-inflammatory-response-related gene expressions (*IL-1β, TNF-α*, and *IL-6*) and the phosphorylation levels of IκBα, and p65 in liver and thus reducing hepatic inflammatory response and apoptosis. Overall, the findings indicate that *L. reuteri* provides significant protection against oxidative stress, mitochondrial impairment, inflammatory response and apoptosis caused by DQ in laying hens, and highlight its potential as a therapeutic probiotic for alleviating oxidative stress and mitochondrial dysfunction to prolong the health of aging poultry.

Introduction

The poultry industry, particularly the egg-laying sector, aims to extend the production cycle of laying hens to enhance overall productivity (Bain et al., 2016). However, a critical challenge in achieving this goal is the prevalence of oxidative stress, especially during post-peaking laying period, which can severely impact animal health and production efficiency (Lv et al., 2024; Wu et al., 2024b). Oxidative stress often occurs in liver of laying hens during the aging process, which generally results in hepatic oxidative damages and mitochondrial dysfunction (Zhao et al., 2024). Accumulating evidence has shown that elevated oxidative stress leads to inflammation and hepatocyte apoptosis during the aging process (Wu et al., 2023; Chen et al., 2021a). The oxidative impairment and dysfunction of liver accompanies by declines in antioxidant capacity and follicular development, which directly decreases egg production and results in economic losses (Moradi et al., 2013). Therefore, it is essential to explore ways to prolong the liver health of aging hens to ensure the durability of egg production.

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Lactobacillus (*L.*) is a genus of beneficial probiotic bacteria, under which there are many probiotic bacteria with antioxidant and anti-inflammatory functions that can promote animal health and performance (Wang et al., 2021). *Lactobacillus* has demonstrated significant efficacy in reducing oxidative damage and enhancing poultry health (Khalique et al., 2019; Xu et al., 2025). Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important regulator of free radical homeostasis, which plays a crucial role in protecting cells against oxidative damage by promoting the expression of antioxidant genes (Kim et al., 2010). A previous study has proven that *Lactobacillus plantarum* 4-2 could alleviate oxidative stress via Keap1-Nrf2 pathway in hens (Xu et al., 2025). Oxidative stress can trigger inflammation through the toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)/nuclear factor-kappa B (NF-κB) pathways, the activation of which has been shown to exacerbate liver damage (Luedde and Schwabe, 2011). *L. johnsonii BS15* has been reported to protect against necrotic enteritis induced hepatic inflammation in broilers by inhibiting NF-κB signaling pathway (Khalique et al., 2019). *L. reuteri*, a species of *Lactobacillus*, has anti-oxidative stress effects, which could alleviate oxidative stress and inflammatory response in animals (Lee et al., 2016; De Marco et al., 2018). Here, we screened a new probiotic strain, *L. reuteri* Y067, which was isolated from the ileum of healthy Lingkun laying hens in China. Our previous data had shown *L. reuteri* Y067 had in vitro anti-inflammatory and antioxidant activities. However, its application in the laying hens remains relatively limited.

The development of an oxidative stress model is important to further address the pathogenesis of hepatic oxidative response and inflammatory responses. Diquat (DQ) is one of the most widely used pyridine herbicides in agriculture in the cultivation of crops, which can enter the cell by diffusion to form unstable DQ^+ and promote the conversion of intracellular molecular oxygen to ROS, such as superoxide anion radicals (Wang et al., 2020). Intraperitoneal injection of DQ is commonly used as an inducer of oxidative damages in poultry, resulting in hepatic oxidative stress and inflammation response (Zha et al., 2023; Wu et al., 2024a). Therefore, a DQ-induced oxidative stress model was established in the present study to investigate the effects of *L. reuteri* Y067 on mitigation of hepatic impairment and dysfunction of aging hens. Specifically, the roles of Nrf2 and NF-κB signaling pathways in the protective effects of *L. reuteri* Y067 were also explored. The findings of the present study can provide a theoretical basis for the application of *L. reuteri* to prolong the liver health of aging hens.

Materials and methods

Animals and experimental design

All procedure of the animal experiments were approved by the Animal Care and Use Committee of Zhejiang University (Hangzhou, China; approval number ZJU20230310). A total of 480 48-week-old Jingbai hens with comparable laying rates (88.2 % ± 1.2 %) were randomly assigned to four groups with eight replicates of 15 hens each. The four groups were control group (Con), *L. reuteri* group (L.R), diquat-challenged group (DQ), and *L. reuteri* supplementation with diquat-challenged group (L.R+DQ). After a two-week acclimation period, hens in Con and DQ groups fed basal diets while hens in L.R and L.R+DQ groups fed diets with 2.0×10^8 CFU/kg of *L. reuteri* Y067. *L. reuteri* Y067, deposited in China Center for Type Culture Collection, was isolated and preserved in our lab. The basal diet consisting of maize and soybean for hens (Table 1) was devised to meet the nutritional parameters of National Research Council (1994).

After 10 weeks of treatment, laying hens in DQ and L.R+DQ groups were injected intraperitoneally with diquat solution (1 mL/kg body weight) (Wu et al., 2024a; Li et al., 2020) while hens in Con and L.R group were injected intraperitoneally with an equivalent amount of 0.90 % saline. Diquat was dissolved in 0.90 % saline to produce a 10 mg/mL solution. Hens had *ad libitum* access to fresh water and mashed experimental diets. The environmental temperature was controlled at around 24°C and the humidity was around 50-60 % with 16 h lightness and 8 h darkness.

Table 1
Ingredients compositions and nutrient levels of the basal diets.

Ingredients, %	Contents	Nutrient levels[2]	Contents
Corn, 7.8%CP	56.00	Metabolic energy, MCal/kg	2.65
Soybean meal, 44%CP	25.50	Crude protein, %	16.50
Wheat middling, 14.5%CP	4.00	Lysine, %	0.84
Emulsified fat powder, 50% Fat	2.00	Methionine, %	0.48
Limestone	9.00	Cysteine + methionine, %	0.76
$CaHPO_4$	1.00	Calcium, %	3.51
Salt	0.30	Total phosphorus, %	0.67
DL-Methionine	0.20	Available phosphorus, %	0.40
Phytase	0.03		
Choline chloride	0.12		
Premix[1]	1.85		
Total	100.00		

[1] The premix provided the following per kg of the diet: vitamin A, 12500 IU; vitamin D3, 4000 IU; vitamin E 80 IU, vitamin K3, 2 mg; vitamin B_{12} 5 mg, thiamine, 1 mg; riboflavin, 8.5 mg; calcium pantothenate, 50 mg; niacin acid, 32.5 mg; pyridoxine, 8 mg; folic acid, 5 mg; iron, 60 mg; copper, 10 mg; manganese, 80 mg; zinc 80 mg; selenium 0.30 mg; iodine 0.3 mg; and antioxidant, 2.00 mg.

[2] Metabolizable energy and available phosphorus contents were calculated values based on the Chinese Feed Composition and Nutritional Value Table (in Chinese, 32th edition, 2021), while the others were measured values.

Sample collection

After 7 days of DQ exposure, one hen from each replicate ($n = 8$) were selected and euthanized, then blood was collected intravenously and centrifuged at 4000 rpm at 4 ℃ for 15 min to isolate the serum for preservation. The abdominal fat and liver were separated and weighed in situ and organ indexes were calculated. A portion of the liver tissue were fixed and the rest of the liver tissues were collected and stored at −80 ℃ for analysis.

Liver health parameters in serum

The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and the contents of triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) in serum were measured using a biochemical analyzer (Randox, London, UK) as previously described (Liu et al., 2021b).

Antioxidant capacity assay

The frozen liver tissue was thawed, 0.1 g of liver tissue was cut and homogenized in 0.9 mL of chilled buffer using a homogenizer as described previously (Liu et al., 2023). The homogenates were then centrifuged at 4,000 rpm for 10 min, and the supernatant was aspirated for analysis. The activities of total antioxidant capacity (T-AOC; kit number: A015-1-2), superoxide dismutase (SOD; kit number: A001-1-2), and glutathione peroxidase (GSH-Px; kit number: A005-1-2) and the content of malonaldehyde (MDA; kit number: A003-1-2) in serum and liver homogenate were assessed using the commercial kits (Nanjing Jiancheng Bioengineering Institute, China).

Liver morphology analysis

Thin slices of liver tissue were stained with hematoxylin and eosin (H&E) and Oil red O (ORO) and obtained digital photographs using an Olympus microsystem (Tokyo, Japan) as previously described (Lv et al.,

2024). The liver fixed in 2.5 % glutaraldehyde were prepared for observation using transmission electron microscopy (JEOL-JEM-1200EX, Peabody, Massachusetts, USA).

Immunofluorescence (IF) staining and TUNEL assay

Immunofluorescence staining was performed as previously described (Liu et al., 2025). Fresh liver tissues were fixed with 4 % paraformaldehyde, rinsed with PBS and dehydrated with 30 % sucrose, then placed in OCT embedding and frozen cut into 5-10 μm sections. After rewarming, the sections were permeabilized with Triton X-100 and closed with normal serum, then sequentially incubated with primary antibody and fluorescent labeled secondary antibody, washed with PBS and sealed with DAPI-containing slices, and observed under fluorescence microscope (BX-61, Olympus, Center Valley, Pennsylvania, USA). TUNEL staining was performed by fixation, permeabilization treatment, and labeling of DNA breaks with TdT enzyme and fluorescently labeled dUTP. After incubation, washing, and nuclear staining, apoptotic signals were observed under a fluorescence microscope.

RT-qPCR analysis for gene expression

The RNA in liver were extracted by using FreeZol Reagent kit, and then generated cDNA by reverse transcription with cDNA synthesis kit (Vazyme, China). RT-qPCR analysis was conducted by using Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) with Taq Pro Universal SYBR qPCR Master Mix reagent (Vazyme, China). The primers are presented in Table S1. The relative expression of mRNA was calculated by $2^{-\Delta\Delta CT}$ method using β-actin as internal reference.

Isolation of hepatic mitochondria

Liver mitochondria were extracted by using mitochondrial isolation kit (Beyotime Biotechnology Institute, Shanghai, China) as we previously described (Liu et al., 2021a). Briefly, the liver tissue was cut into small pieces and then homogenized in pre-chilled mitochondrial extraction buffer. The homogenate was then subjected to low-speed centrifugation at 1,000-2,000 g to remove cell debris and nuclei. The obtained supernatant was further centrifuged at 10,000-12,000 g to pellet the mitochondria and then resuspended in cold extraction buffer and washed by another high-speed centrifugation. Finally, the purified mitochondria were resuspended in an appropriate buffer for subsequent experiments.

ROS Determination

The ROS in liver mitochondria was determined by dichlorohydrofluorescein diacetate method as we previously described (Liu et al., 2021a). Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The arbitrary units of dichlorofluorescein (DCF) fluorescence intensity were used to indicate ROS levels in samples.

Mitochondrial membrane potential analysis

The mitochondrial membrane potential was measured using the commercial assay kit with fluorescent dye JC-1 (Beyotime Institute Biotechnology, Shanghai, China). Briefly, the isolated liver mitochondria were mixed with the JC-1 staining solution, and fluorescence intensity was measured using an automatic fluorescence microplate reader. The mitochondrial membrane potential was expressed as the red/green fluorescence intensity ratio.

Statistical analysis

All data were analyzed by using SPSS software (version 26.0; SPSS Inc., Chicago, IL, USA) and expressed as mean ± standard deviation (SD). Data pertaining to the organ index, serum biochemical parameters, antioxidant capacity, and gene expressions were analyzed by using the GLM procedure as a 2 (*Lactobacillus reuteri* supplementation or not) × 2 (DQ exposure or not) factorial design. Student's t-test was used for the analysis of differences between the two groups. Graphs were visualized utilizing GraphPad prism software (version 8.0; GraphPad Software Inc., San Diego, CA, USA).

Results

L. reuteri improved the metabolic phenotypes associated with oxidative stress induced by diquat

The representative gross appearance and H&E staining images of the liver are presented in Fig. 1. Compared to the Con, no differences were observed in metabolic phenotypes of *L. reuteri* treated hens except for ALT activity in serum ($P > 0.05$). The phenotypic images and H&E staining of livers in DQ group displayed liver hypertrophy, enlarged, yellowish livers with considerable fat vacuoles and inflammatory cell infiltration as compared with the control hens (Fig. 1a and b). In addition, the liver index as well as ALT and AST activity increased significantly ($P < 0.05$) in DQ-challenged hens (Fig. 1c-f). However, *L. reuteri* pretreatment resulted in a significant alleviation evidenced by the decreased lipid vacuoles and inflammatory infiltration in the H&E sections, decreased liver index and ALT and AST activities compared to the DQ-challenged hens ($P < 0.05$). These results collectively indicated that *L. reuteri* could improve the metabolic phenotypes associated with oxidative stress induced by diquat.

L. reuteri alleviated hepatic lipid metabolism dysfunction

The representative oil red O (ORO) staining images and serum lipid metabolism parameters are shown in Fig. 2. Compared with the Con, *L. reuteri* decreased TG and increased HLD-C levels in serum ($P < 0.05$). DQ exposure increased the area of red lipid droplets in ORO and resulted in hepatic steatosis (Fig. 2a). This observation was further verified by the increased TC, TG and LDL-C contents in serum of DQ-challenged hens (Fig. 2c-f). However, these effects can be effectively reduced by dietary *L. reuteri* intervention. Compared with DQ, *L. reuteri* intervention decreased the area of red lipid droplets in ORO staining (Fig. 2a), as well as decreased abdominal fat percentage (Fig. 2b), the TC, TG and LDL-C levels in serum (Fig. 2d-f, $P < 0.05$). Overall, *L. reuteri* positively influenced lipid metabolism and alleviated hepatic lipid metabolism dysfunction induced by DQ challenge in laying hens (Fig. 2c–f).

L. reuteri attenuated diquat-induced oxidative stress

As shown in Fig. 3, compared with the Con, *L. reuteri* increased the activity of T-AOC, GSH-Px and SOD ($P < 0.05$) in serum and liver while DQ exposure decreased the activity of T-AOC, GSH-Px and SOD in both serum and liver (Fig. 3a and b). This observation was further verified by the decreased MDA contents in *L. reuteri* treated hens and increased MDA contents in DQ-challenged hens ($P < 0.05$). However, these negative effects induced by DQ exposure can be effectively reduced by dietary *L. reuteri* intervention. Dietary *L. reuteri* supplementation increased the activity of T-AOC, GSH-Px, and SOD in serum as well as T-AOC and SOD in liver of hens challenged by diquat ($P < 0.05$).

L. reuteri mitigated hepatic mitochondrial oxidative damages and dysfunction

As shown in Fig. 4a, transmission electron microscopy of liver mitochondria showed that diquat challenge resulted in a decrease in the number of mitochondria, an enlargement of the remaining mitochondria, and the reduction or rupture of intra-mitochondrial cristae.

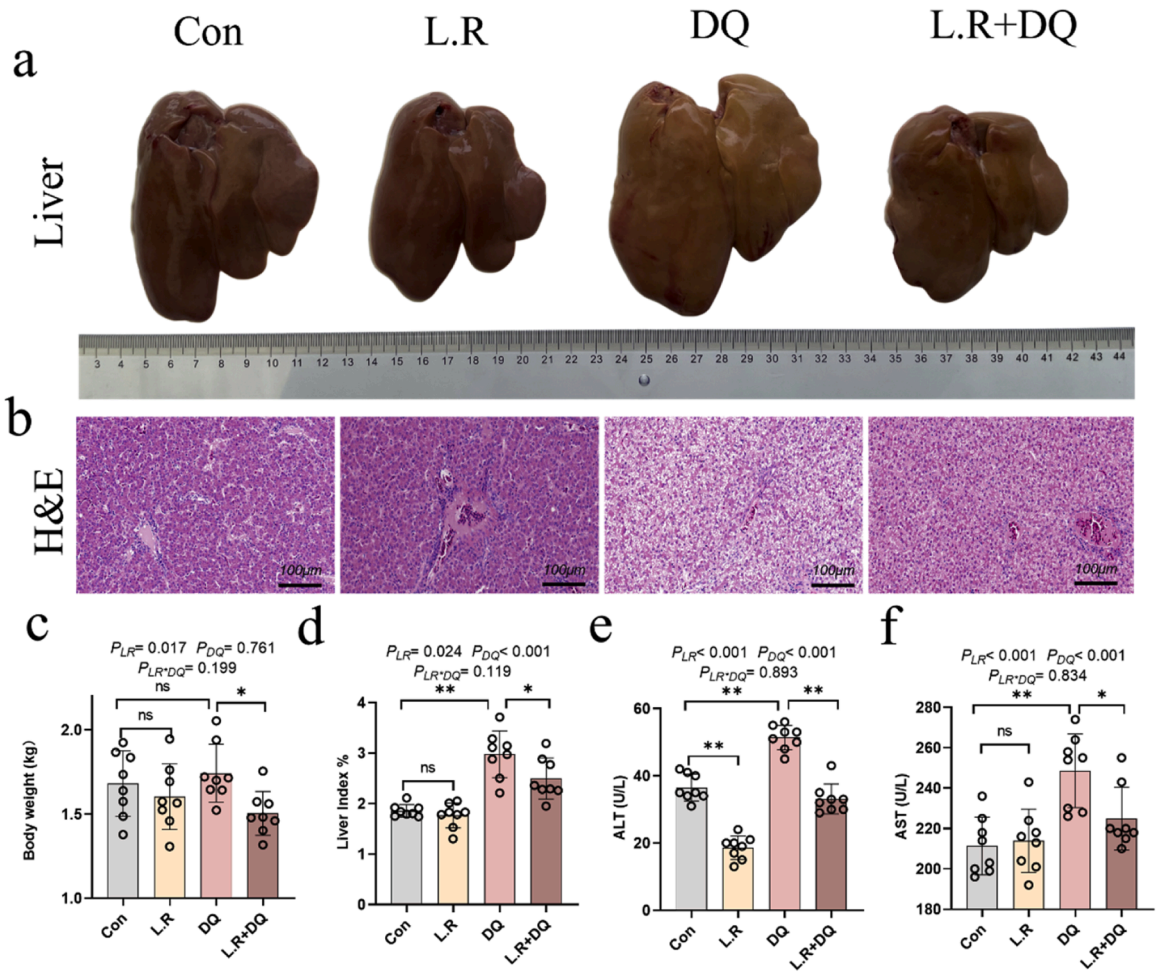

Fig. 1. Effect of *L. reuteri* on the metabolic phenotypes associated with oxidative stress induced by diquat. **a.** Gross specimens of livers; **b.** Representative photomicrographs of liver sections stained with H&E (100 um); **c.** Mean body weight; **d.** Liver index; **e-f.** Serum AST and ALT activity. AST, aspartate aminotransferase; ALT, alanine aminotransferase. The results were represented by mean ± SD. * $P < 0.05$, ** $P < 0.01$.

Mitochondrial ROS production significantly elevated while mitochondrial membrane potential (MMP) and mtRNA content diminished following DQ challenge ($P < 0.05$, Fig. 4b and c). Additionally, DQ exposure downregulated the mRNA expressions of mitochondrial function-related genes such as *NRF1, TFAM, PGC-1α, SIRT1*, and *POLMRT*. Conversely, *L. reuteri* powerfully combated the harmful effects of oxidative stress and thus preserving mitochondrial integrity. The MMP levels were improved after supplementing with *L. reuteri* ($P < 0.05$). The mRNA expressions of mitochondrial function-related genes such as *NRF1, PGC-1α, SIRT1*, and *POLMRT* mRNA levels were upregulated after administering *L. reuteri* as compared with DQ ($P < 0.05$, Fig. 4e).

L. reuteri attenuated hepatic oxidative injury and dysfunction via activating Nrf2/HO-1 pathways

As shown in Fig. 5, compared to the Con, *L. reuteri* supplementation upregulated the mRNA expression levels of oxidative stress related gens such as *Nrf2, HO-1, NQO1*, and *SOD1* ($P < 0.05$, Fig. 5a). DQ exposure downregulated the gene expression levels of *Nrf2, SOD1* and *GPX4* ($P < 0.05$). However, dietary *L. reuteri* increased the expression of *Nrf2, HO-1, NQO1, SOD1*, and *GPX4* compared to DQ ($P < 0.05$). This observation was further verified by the increased protein expressions of the key protein Nrf2 and HO-1 in liver of L.*R*+DQ-treated hens determined by using immunofluorescence ($P < 0.05$, Fig. 5b and c).

L. reuteri reduced hepatic inflammatory response

As shown in Fig. 6, compared to the Con, *L. reuteri* supplementation decreased the mRNA expression levels and contents of the pro-inflammatory IL-1β, TNF-α, and IL-6 and increased the anti-inflammatory IL-10 mRNA expression levels and contents in liver ($P < 0.05$). DQ challenge increased the gene and protein expression levels of IL-1β, TNF-α, and IL-6 in liver. However, dietary *L. reuteri* supplementation decreased the gene expression levels of *IL-1β, TNF-α*, and *IL-6* and decreased the protein expression IL-1β and TNF-α in liver of DQ-treated hens ($P < 0.05$).

L. reuteri decreased hepatic apoptosis

As shown in Fig. 7a and b, the TUNEL staining result revealed that dietary *L. reuteri* exhibited a lower green fluorescence intensity than the control hens ($P < 0.05$). Correspondingly, the *Bcl-2* mRNA level of was remarkably upregulated by *L. reuteri* treatment ($P < 0.05$, Fig. 7c). DQ exposure increased green fluorescence intensity of TUNEL staining. However, the green fluorescence intensity was markedly decreased after *L. reuteri* supplementation in comparison to the DQ group ($P < 0.05$). Correspondingly, *L. reuteri* decreased *Bax* and *Caspase-3* mRNA expression while upregulated *Bcl-2* mRNA expression in the DQ+*L*.R group ($P < 0.05$). This modulation suggests a protective effect against apoptosis triggered by DQ-induced oxidative stress and inflammatory response.

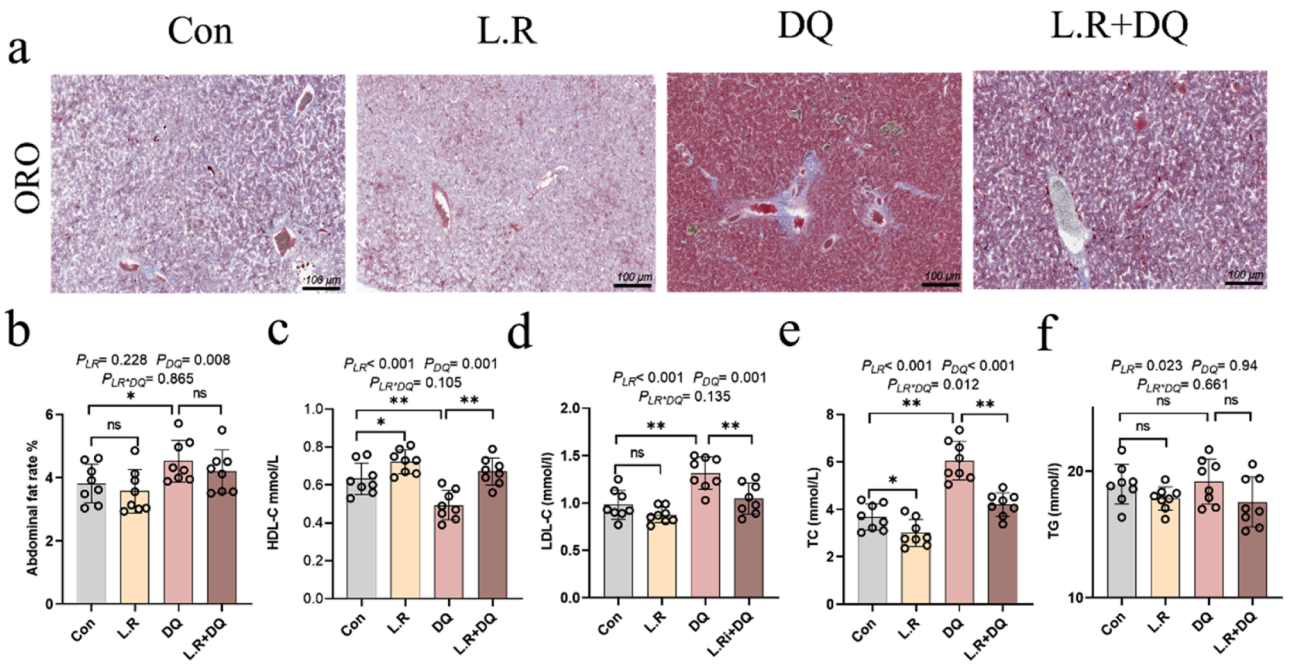

Fig. 2. Effect of *L. reuteri* on hepatic lipid metabolism in laying hens. **a.** Representative images of photomicrographs of fixed liver sections after staining with ORO (100 μm); **b**. Quantification of the abdominal fat rate; **c-f**. Serum HDL-C, LDL-C, TC and TG contents. HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride. The results were represented by mean ± SD. * $P < 0.05$, ** $P < 0.01$.

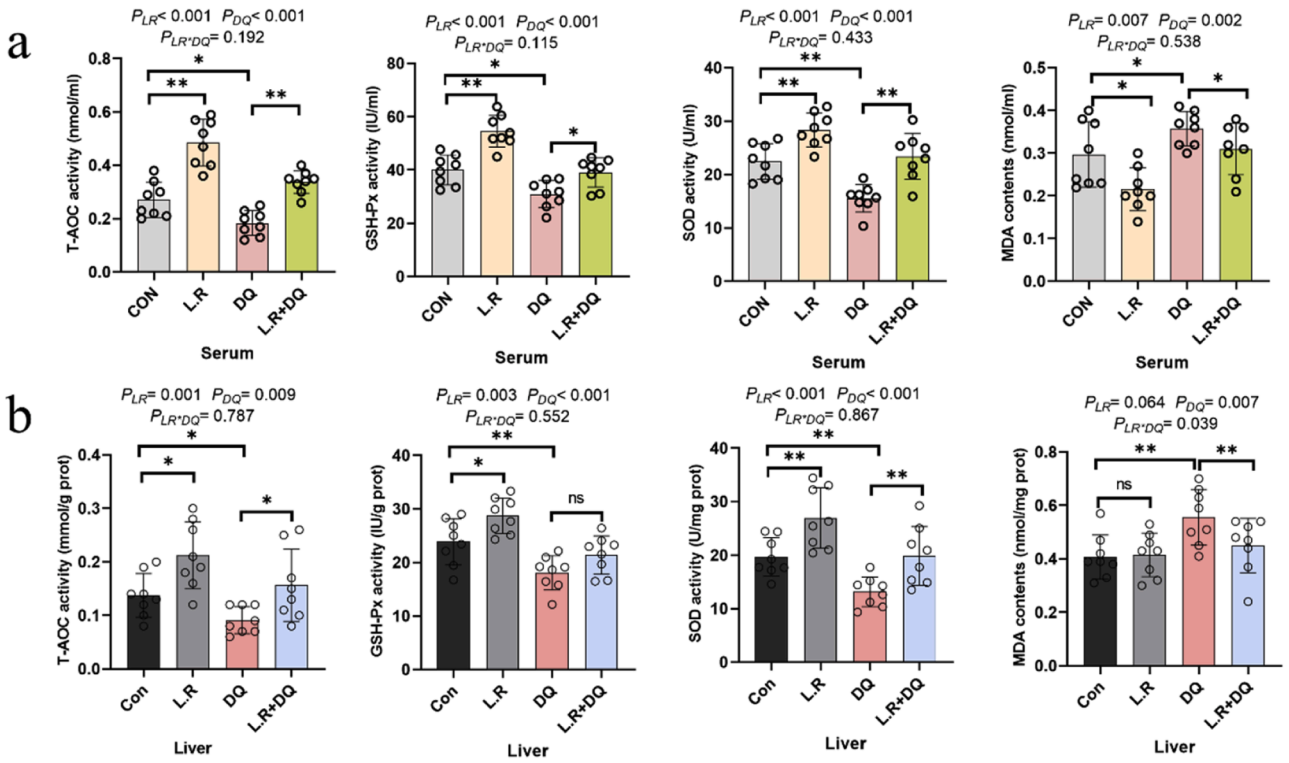

Fig. 3. Effect of *L. reuteri* on the serum and hepatic antioxidative enzyme activities. a. T-AOC, GSH-Px, and SOD activity and MDA contents in serum; b. T-AOC, GSH-Px, and SOD activity and MDA contents in liver. T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, malonaldehyde. The results were represented by mean ± SD. *$P < 0.05$, **$P < 0.01$.

L. reuteri attenuated hepatic inflammatory response and apoptosis via suppression of NF-κB signaling pathways

As shown in Fig. 8a, the mRNA expression levels of *TLR4, MYD88, NF-κB, IκBα* in liver tissues significantly upregulated by DQ challenge ($P < 0.05$), while *L. reuteri* downregulated the mRNA expression levels of the key genes *TLR4, MYD88*, and *NF-κB*. This observation was further verified by the decreased protein expressions of the key protein TLR4, NF-κB p-p65, and p-IκBα in liver of L.*R*+DQ-treated hens determined by using immunofluorescence ($P < 0.05$, Fig. 5b and c).

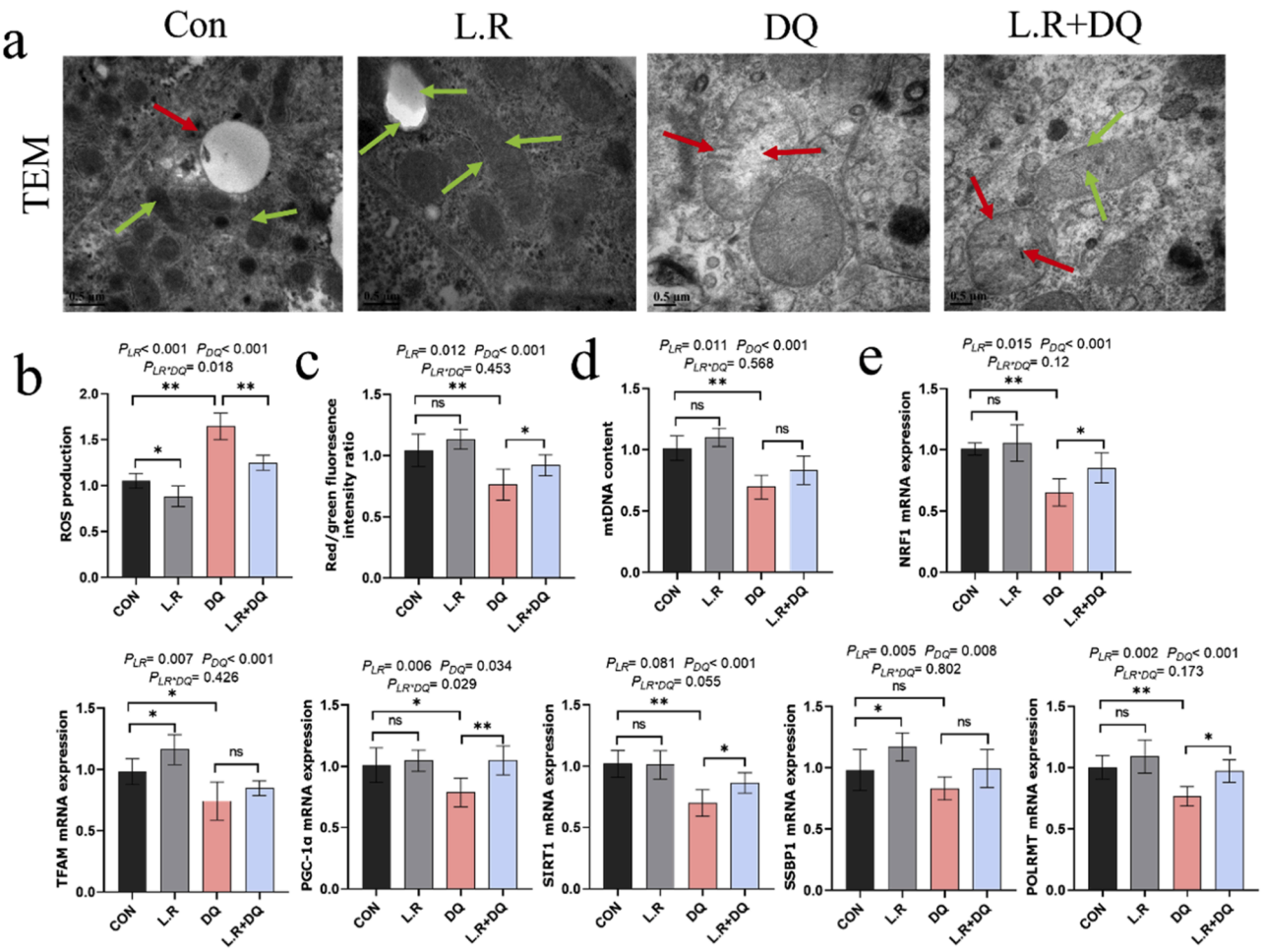

Fig. 4. *Lactobacillus reuteri* mitigated hepatic mitochondrial damage caused by diquat challenge. **a**. Transmission electron microscopy of liver mitochondria; **b**. hepatic mitochondrial ROS production; **c**. mitochondrial membrane potential in liver; **d**. mitochondrial DNA (mtDNA) content in the liver; **e**. mRNA expressions of mitochondrial function-related genes. *NRF1*, nuclear respiratory factor 1; *TFAM*, transcription factor A, mitochondrial; *PGC-1α*, peroxisome proliferative activated receptor gamma coactivator 1 alpha; *SIRT1*, sirtuin 1; *SSBP1*,single stranded DNA binding protein 1; *POLRMT*, DNA-directed RNA polymerase, mitochondrial. The results were represented by mean ± SD. * $P < 0.05$, ** $P < 0.01$.

Discussion

Aging in laying hens is closely related to events comprising cytoarchitectural lesions, oxidative stress, inflammatory damage, and lipid disorders in the liver, especially in the late egg-laying stage (Xie et al., 2019; Chen et al., 2021b). Oxidative stress accumulation and inflammation responses can do harm to overall health of laying hens, which could decline egg production and disrupt the natural egg-laying cycle (Costantini et al., 2016). Thus, the alleviation of oxidative stress and inflammatory responses in liver is one of the most important targets for improving the performance of hens. The development of an oxidative stress model is important to further address the pathogenesis of hepatic oxidative and inflammatory responses. Diquat has been used to establish liver oxidative stress models in broilers and pigs (Wu et al., 2024a; Li et al., 2022). In this study, intraperitoneal injection of DQ were used to successfully establish a hepatic oxidative stress model in hens evidenced by the elevated ROS levels, lipid over-accumulation, liver index, and inflammatory responses as well as the decreased mtDNA contents and antioxidant enzyme activities in liver. In parallel with the increased liver index and lipid accumulation, the AST and ALT activities and especially TC contents and ORO staining aeras were significantly raised in DQ-treated hens, suggesting that DQ-induced oxidative stress resulted in liver dysfunction, which is further supported by the disrupted redox balance.

L. reuteri is an effective *Lactobacillus* species found in the digestive tract (Hou et al., 2015). Growing evidence has shown *L. reuteri* exhibits antioxidant, anti-microbial, anti-inflammatory, and hepatoprotective properties, which is essential for safeguarding animals against oxidative stress and inflammatory responses (Ding et al., 2024; Hu et al., 2021). In the present study, *L. reuteri* alleviated DQ-induced liver oxidative injury and improved mitochondrial function by increasing the activities of antioxidant enzymes and restraining inflammatory responses and apoptosis. In consistent with the improved antioxidant capacity, *L. reuteri* decreased AST and ALT activities and normalized the apoptotic gene mRNA levels in DQ-treated hens. Moreover, the liver index and TC content in liver of *L. reuteri*-treated hens challenged by DQ were reduced to a value similar to that of the normal controls. Overall, these compelling findings clearly highlight the protective benefits of *L. reuteri* against oxidative stress in hens exposed to diquat and provided new evidence regarding the potential therapeutic effects on hepatic oxidative damages.

Previous studies have reported that DQ exposure declined the activities of SOD and GSH-Px, and increased MDA contents in serum and the liver of broilers (Chen et al., 2021b; Zha et al., 2023). In our study, DQ exposure decreased the activities of T-AOC, GSH-Px and SOD in liver, which is partially in agreement with the aforementioned studies. These results together suggest that DQ exposure disrupted the redox balance by inhibiting antioxidant enzymes activity and increasing lipid peroxidation. Previous studies in broilers (Chai et al., 2023) and laying hens (Xu et al., 2025) have confirmed that *Lactobacillus* could scavenge

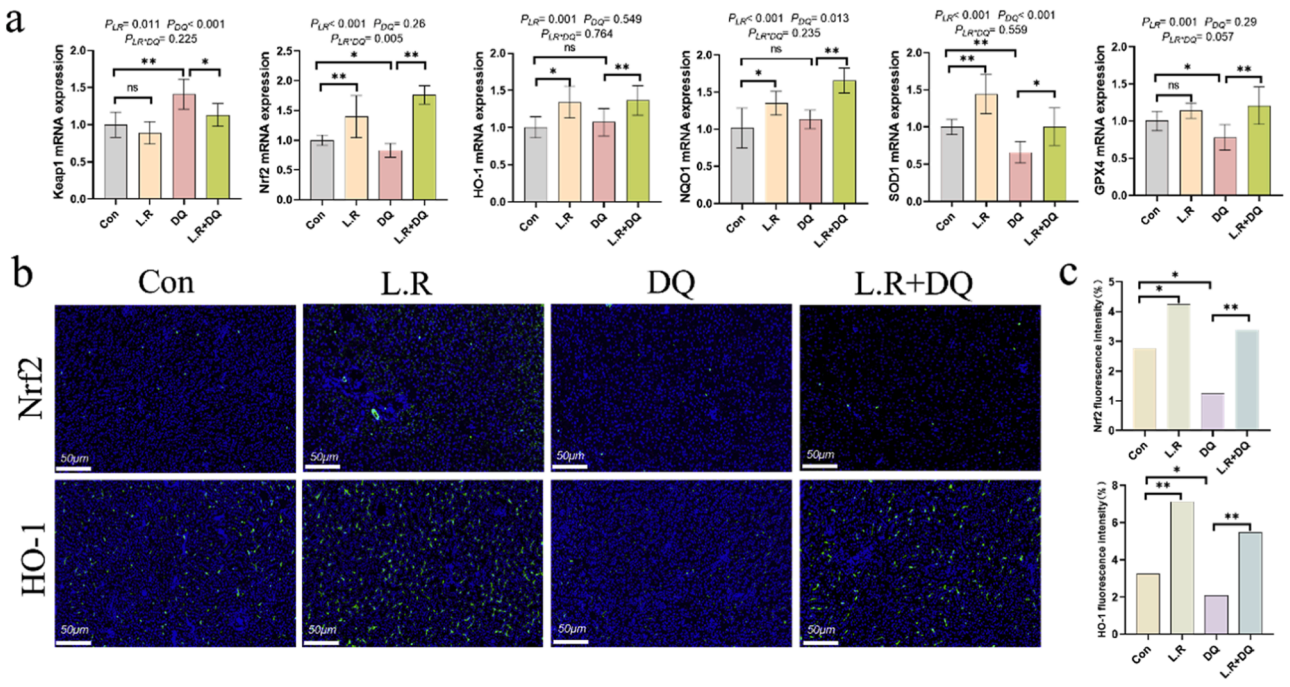

Fig. 5. *L. reuteri* attenuated diquat-induced hepatic oxidative damage through activating keap1-Nrf2/HO-1 signaling pathways. A. The mRNA levels of antioxidant-related genes in the liver. B. Representative immunofluorescence staining images of Nrf2 and HO-1 in hepatic tissue. **C.** The relative fluorescence intensity of Nrf2 and HO-1. The results were represented by mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

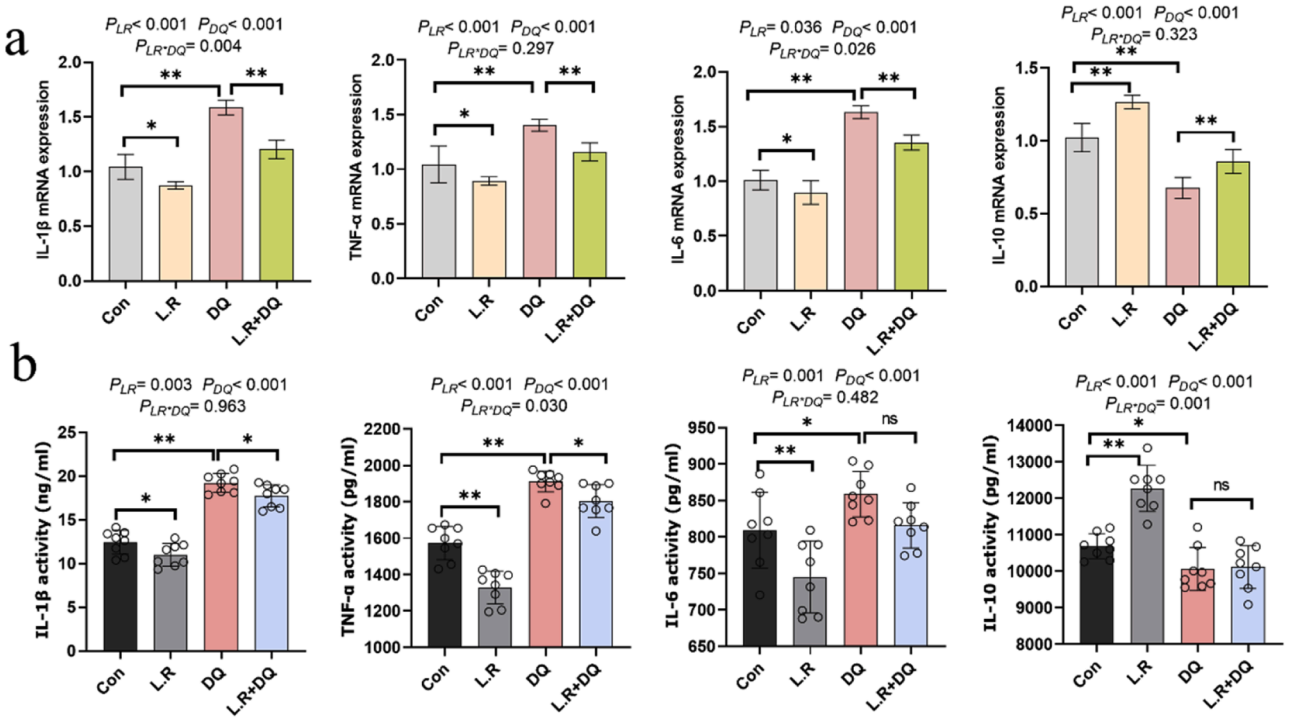

Fig. 6. Effect of dierary *L. reuteri* on hepatic inflammatory response of DQ-challenged hens. **a**. Relative mRNA expression levels of inflammatory cytokine *TNF-α, IL-1β, IL-6* and *IL-10* in liver; **b**. The levels of inflammatory cytokine TNF-α, IL-1β, IL-6 and IL-10 in liver.

ROS and enhance antioxidant capacity. In this study, dietary *L. reuteri* reversed the redox imbalance evidenced by the increased activities of T-AOC, SOD, and GSH-Px in serum as well as T-AOC and SOD in liver. These findings together indicated that *L. reuteri* could alleviate DQ-induced liver oxidative damages by increasing the antioxidant capacity and eliminating oxygen free radicals.

Mitochondria are continuously exposed to ROS and therefore susceptible to oxidative stress (Mikhed et al., 2015). Growing evidence suggests that mitochondrial DNA damage is associated with impaired redox homeostasis (Georgieva et al., 2017). The overproduction of ROS induced by oxidative stress results in the opening of anion channels within the inner mitochondrial membrane and initiates mitochondrial

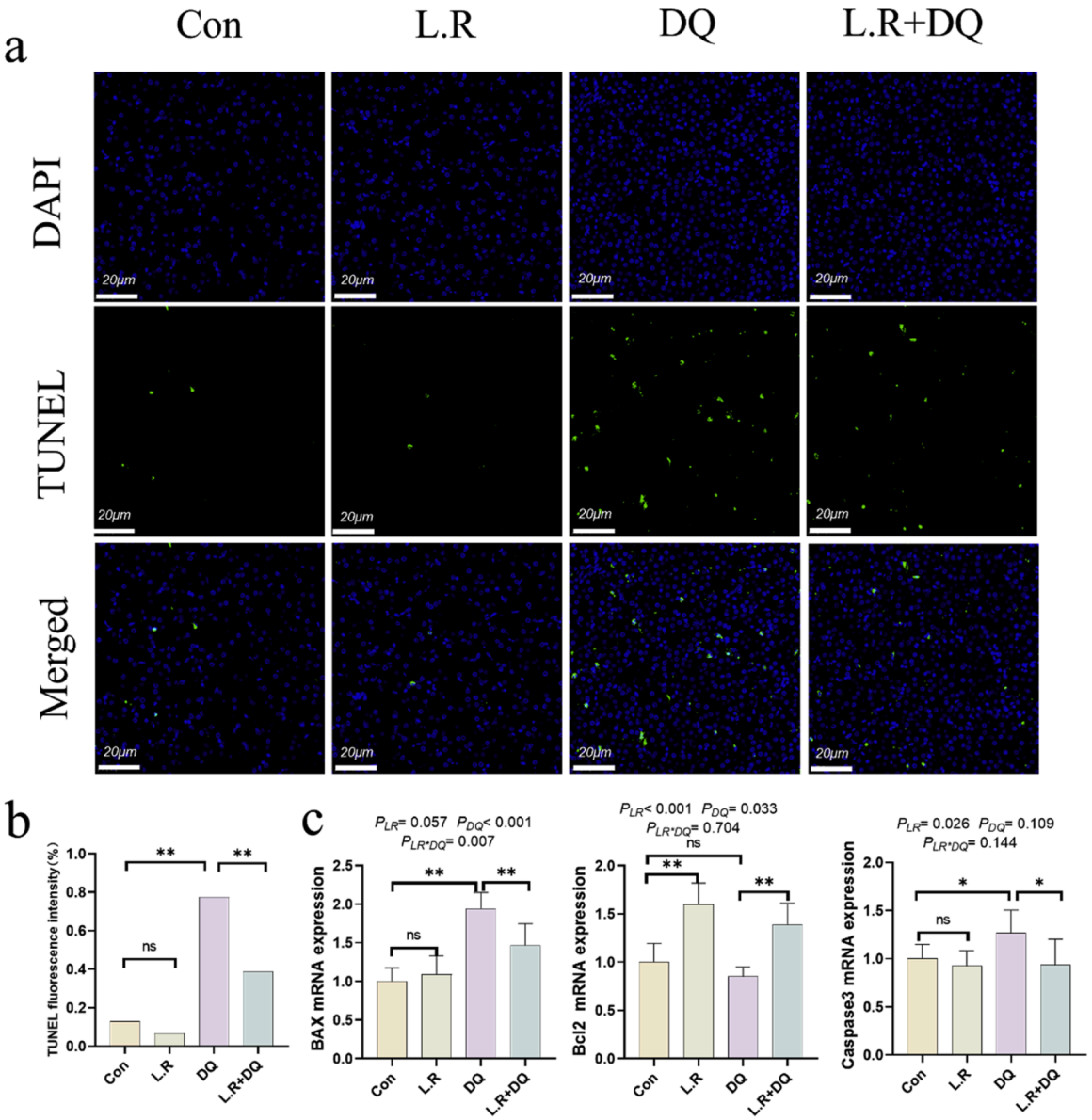

Fig. 7. *L. reuteri* decreased hepatic cells apoptosis in diquat-induced oxidative stress. a. Representative TUNEL immunofluorescence staining images in hepatic tissue; b. TUNEL fluorescence intensity; c. The mRNA levels of apoptosis-related genes. The results were represented by mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

membrane depolarization, leading to a reduction in mitochondrial membrane potential (Mikhed et al., 2015). In alignment with the findings of previous studies (Cao et al., 2018; Zhang et al., 2020), our data showed an elevation in ROS content and a reduction in membrane potential and mtDNA content within the DQ group. Nevertheless, this was found to be mitigated in the group that received *L. reuteri* supplementation. *Lactobacillus* has been reported to modulated membrane potential and mtDNA content. For instance, *L. salivarius* supplement increased the mitochondrial membrane potential in liver of the geese (Qiu et al., 2024). *L. plantarum* P8 supplementation decrease ROS levels and downregulated mtDNA copy number in breast muscle of broilers (Yuan et al., 2023). These findings suggest that *Lactobacillus* may improve mitochondrial membrane potential and decrease ROS levels, which positively recovered mtDNA content.

The alterations in mtDNA levels were associated with mitochondrial biogenesis related genes (Lee and Wei, 2005). Mitochondrial biosynthesis requires the coordinated function of the nuclear genome and mitochondrial genome (Giegé et al., 2005). *PGC-1α* serves as a pivotal transcription factor that regulates mitochondrial biosynthesis and energy metabolism, orchestrating the cellular response to energy demands. *NRF1* and *TFAM* maintain mitochondrial function by facilitating the replication and transcription of mtDNA (Taherzadeh-Fard et al., 2011). SSBP1 *is* primarily engaged in the maintenance of DNA integrity and stability, whereas POLRMT is the primary enzyme mediating mitochondrial DNA transcription (Rusecka et al., 2018). This study indicated that DQ-induced oxidative stress downregulated the mRNA expression of several key transcription factor genes in the liver, including *PGC- 1α, NRF1, TFAM, SIRT1, SSBP1* and *POLRMT*, which is partly consisting with the findings of a previous study in piglets (Cao et al., 2018). Recent studies have demonstrated that *Lactobacillus* exhibits significant protective effects on mitochondrial biosynthesis and energy metabolism. For instance, *L. plantarum* JM113 could alleviate mitochondrial dysfunction induced by deoxynivalenol in the jejunum of broilers (Tong et al., 2025). *L. salivarius* could enhance mitochondria energy metabolism and thus promote the differentiation of ISCs in laying hens (Zhou et al., 2022). Consistent with these findings, *L. reuteri* administration in this study provided significant protection against mitochondrial damage and enhance mitochondrial biogenesis. This may

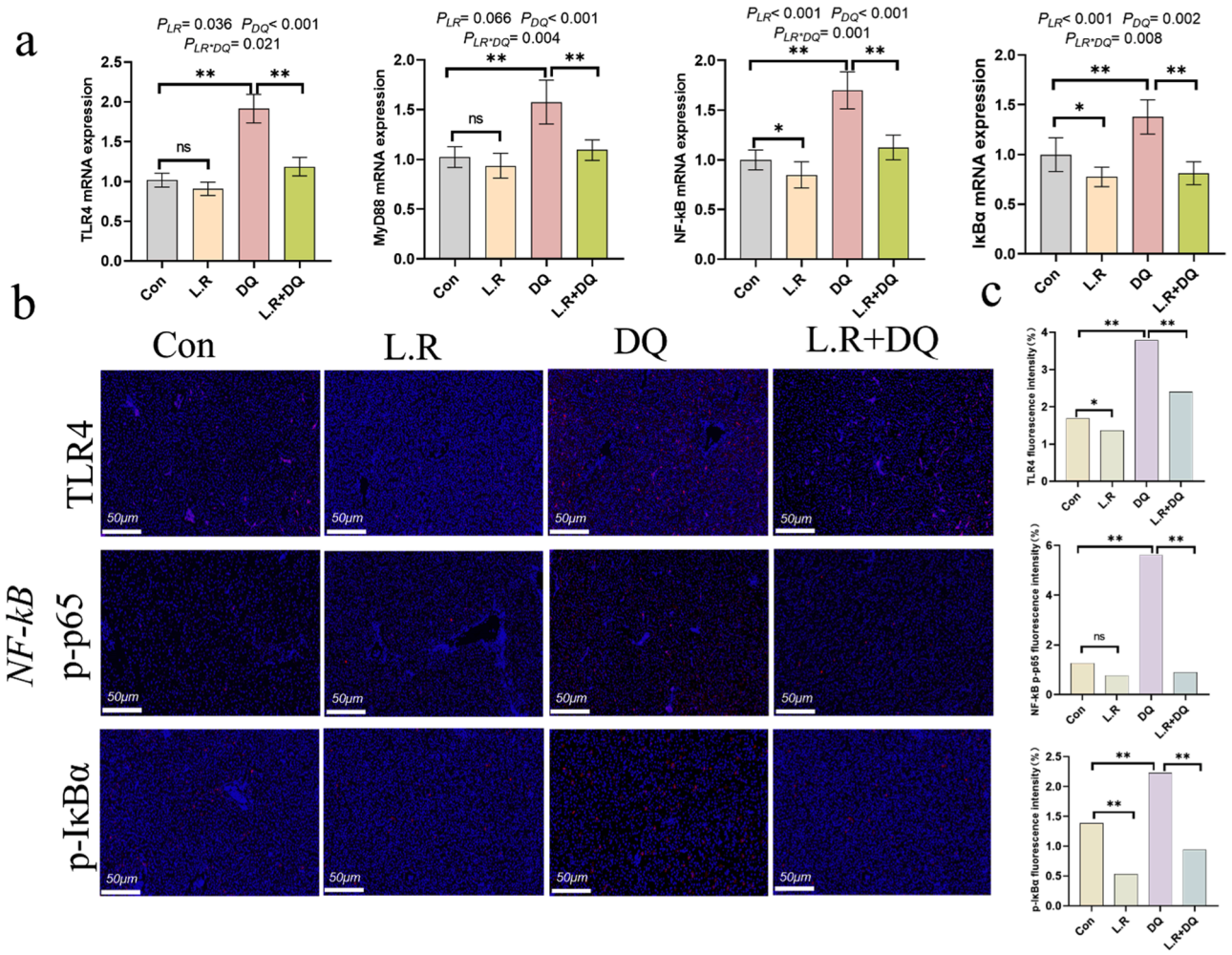

Fig. 8. *L. reuteri* reduced hepatic inflammatory response via inhibiting TLR4/MyD88/NF-κB pathway **a**. The key gene *TLR4, MyD88, NF-κB*, and *IκBα* mRNA expression in liver; **b**. The relative red fluorescence intensity of TLR4, NF-kB p-p65 and p-IκBα in the liver. The results were represented by mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

be explained by the fact that *L. reuteri* curtailed ROS production and mitigated the depletion of membrane potential and thus recovering mitochondrial function.

Nrf2 is known to be mainly responsible for stimulating the gene expression of various antioxidant and detoxification enzymes such as SOD and GPX (Wu et al., 2024a). Nrf2/Keap1/HO-1pathway is an important regulator of free radical homeostasis, which has been reported to play important roles in DQ-induced hepatic oxidative damages and mitochondrial dysfunction (Jayasuriya, 2021; Wu et al., 2024a). During oxidative stress, ROS alter the structure of Keap1 protein, causing it to release Nrf2, which then leaves the cytoplasm and enters the nucleus and regulates downstream antioxidant proteins (Shaw and Chattopadhyay, 2020). HO-1 is a downstream protein of Nrf2 that maintains inner cellular environmental homeostasis to alleviate oxidative stress, maintain redox balance, and prevent mitochondrial damage (Consoli et al., 2021). In this study, DQ exposure upregulated *Keap1* mRNA levels, and decreased the mRNA expressions of *Nrf2* and its downstream gene *SOD1* and *GPX4*. However, *Nrf2, GPX4,* and *SOD1* gene expression in the liver was upregulated after *L. reuteri* intervention and the results were further verified by immunofluorescence of Nrf2 and HO-1, indicating that *L. reuteri* enhanced the downstream antioxidant enzyme activities through the Nrf2/Keap1/HO-1 pathway. Similarly, a previous study also reported that *Lactobacillus plantarum* 4-2 could alleviate oxidative stress in hens via Keap1-Nrf2 pathway (Xu et al., 2025), suggesting that *L. reuteri* has a strong potential in promoting cellular resilience.

Oxidative stress can trigger inflammation through the TLR4/NF-κB or NLRP-3 pathways, resulting in the pro-inflammatory cytokines generation (Pereda et al., 2006). In this study, DQ exposure induced inflammatory reactions evidenced by the increased IL-1β, IL-6, and TNF-α contents in liver, which was in agreement with the findings of a previous study (Zha et al., 2023). *L. reuteri* treatment effectively decreased IL-1β and TNF-α contents in liver, which were consisted with previous studies conducted in broilers that reported *L. reuteri* could maintain immune homeostasis against LPS-induced inflammation (Hu et al., 2021; Wu et al., 2024a). The TLR4/MYD88/NF-κB signaling pathway is a vital immune pathway linked to anti-inflammatory responses (Zhu et al., 2014). TLR4 recognizes molecules from pathogens or damage, recruits MyD88, and activates the IκB kinase (IKK) complex, which causes IκB degradation and release *NF-κB* dimers (like *p65/p50*) to trigger the transcription of inflammatory genes (Bakkar and Guttridge, 2010). Our data showed that DQ activated TLR4/MYD88/NF-κB pathway and upregulated the mRNA expression of downstream gene *IL-1β, IL-6*, and *TNF-α*, and thus ultimately leading to inflammatory response exacerbated liver damages. The modulation of NF-kB pathway to inhibit inflammation by *Lactobacillus* has been reported in previous studies. For instance, *L.salivarius* or *L. reuteri* blocked the interaction of LPS and TLR4 and inhibited the transduction of MyD88-NF-kB to play anti-inflammatory effects in hens (Xu et al., 2022) and broilers (Hu et al., 2021). Consistently, *L. reuteri* reversed these changes and suppressed

crucial TLR4/MYD88/NF-κB pathway in this study.

Oxidative stress and inflammatory infiltration within the liver can also result in increased hepatocyte apoptosis, which in turn promoted liver epithelial cell loss (Guicciardi et al., 2013). In addition, impaired mitochondria generated more ROS can also trigger apoptosis (Sastre et al., 2000). Caspase-3, Bax, and Bcl-2 are genes playing an important regulatory role in the process of apoptosis (Wu et al., 2024a). These genes could influence the antioxidant balance and redox environment within the mitochondria to regulate mitochondrial DNA damage and cell death (Cao et al., 2016). A previous study has reported that DQ exposure might upregulate *Caspase-3* expression and induce apoptotic hepatocytes in the liver of broilers (Chen et al., 2020). Our investigation also showed *Bax* and *Caspase-3* mRNA levels increased while the anti-apoptotic factor *Bcl-2* mRNA levels decreased following DQ injection. This alteration in the expression of apoptotic regulators indicated that DQ induces a shift toward a pro-apoptotic environment within the hepatic tissue, contributing to cell death and exacerbating liver injury associated with oxidative stress. *Lactobacillus plantarum* 16 has been reported to inhibit intestinal apoptosis in broilers by elevating *Bcl-2* mRNA expression while decreasing *Bax* and *Caspase-3* mRNA expression

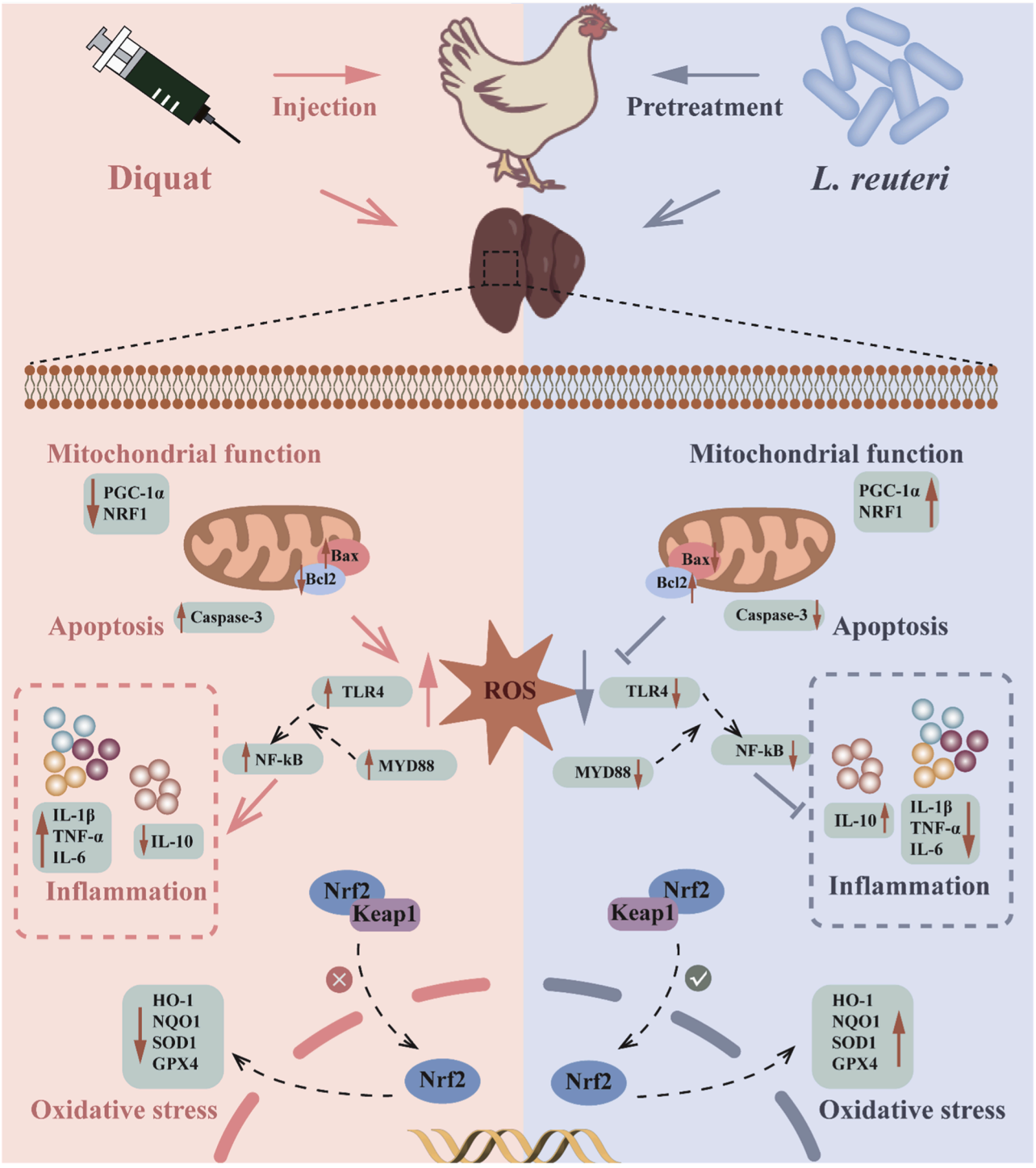

Fig. 9. *Lactobacillus reuteri* alleviates diquat induced hepatic impairment and dysfunction via activation of the Nrf2 antioxidant system and suppression of NF-κB mediated inflammatory response and apoptosis.

(Wu et al., 2019). Dietary *L. reuteri* could prevent from oxidative stress and inflammation mediated hepatic apoptosis (Lin et al., 2023; Ding et al., 2024). In agreement with the aforementioned studies, *L. reuteri* markedly reduced the number of TUNEL positive cells, a key indicator of apoptosis, suggesting that *L. reuteri* exerted a protective effect against apoptosis in the liver. The decreased mRNA expression of *Bax* and *Caspase-3* further verified it. These results strongly proved that *L. reuteri* could protect hepatocyte from apoptosis, thereby offering a promising strategy for alleviating oxidative stress-related liver injuries in poultry.

Conclusions

In summary, *L. reuteri* could alleviate DQ-induced oxidative stress, mitochondrial dysfunction, inflammatory responses and apoptosis in the liver. *L. reuteri* may maintain mitochondrial function and alleviate oxidative stress of liver by activation of Keap1-Nrf2/HO-1 pathway mediated antioxidant system. *L. reuteri* supplementation could also inhibit hepatic TLR4/MyD88/NF-κB pathway activation, and thus reducing hepatic inflammatory response and apoptosis (Fig. 9). These results imply that *L. reuteri* may serve as a potential supplement for the prevention and management of liver oxidative damages and mitochondrial dysfunction to prolong the health of aging poultry.

Ethics approval

All animal experiments were conducted in strict accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Zhejiang University (Hangzhou, China; approval number ZJU20230310).

Declaration of competing interest

We have no conflict of interest for the manuscript to be submitted for publication in this journal, and there has been no significant financial support for this work that could have influenced its outcome.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.104997.

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