

Lactobacillus plantarum KSFY06 Prevents Inflammatory Response and Oxidative Stress in Acute Liver Injury Induced by D-Gal/LPS in Mice

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Aim: The purpose of this study is to investigate the preventive effect of *Lactobacillus plantarum* KSFY06 (LP-KSFY06) on D-galactose/lipopolysaccharide (D-Gal/LPS)-induced acute liver injury (ALI) in mice.

Methods: We evaluated the antioxidant capacity of LP-KSFY06 in vitro, detailed the effects of LP-KSFY06 on the organ index, liver function index, biochemical index, cytokines, and related genes, and noted the accompanying pathological changes.

Results: The results clearly showed that LP-KSFY06 can remove 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzthiazoline -6-sulphonic acid) diammonium salt (ABTS) free radicals in vitro. The analysis of the organ index and pathology demonstrated that LP-KSFY06 significantly prevented ALI. Biochemical and molecular biological analysis showed that LP-KSFY06 prevented a decrease in the antioxidant-related levels of superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), and total antioxidant capacity (T-AOC), and also prevented an increase in aspartate aminotransferase (AST), alanine aminotransferase (ALT), malondialdehyde (MDA), myeloperoxidase (MPO), and nitric oxide (NO) levels. LP-KSFY06 upregulated the anti-inflammatory factor interleukin (IL)-10 and downregulated the pro-inflammatory factors IL-6, IL-1 β , tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ). These oxidative and inflammatory indicators were consistent with the results of gene detections. Furthermore, we determined that LP-KSFY06 downregulated Keap1, NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1, nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B), IL-18, and mitogen-activated protein kinase 14 (MAPK14 or p38), upregulated Nrf2, heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase [quinone] 1 (NQO1), B-cell inhibitor- α (I κ B- α), and thioredoxin (Trx) mRNA expression. These may be related to the regulation of the Kelch-like ECH-associated protein-1 (Keap1)-nuclear factor-erythroid-2-related factor (Nrf2)/antioxidant response element (ARE) and NLRP3/NF- κ B pathways.

Conclusion: LP-KSFY06 is an effective multifunctional *Lactobacillus* with strong antioxidant and anti-inflammatory ability that can prevent D-gal/LPS-induced ALI in mice and assist in maintaining health.

Keywords: *Lactobacillus plantarum* KSFY06, D-galactose, lipopolysaccharide, acute liver injury, mice

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Introduction

Acute liver injury (ALI) is a group of clinically common liver disease syndromes resulting from the massive death of hepatocytes. If ALI progresses to liver failure, mortality can then ensue.¹ The prognosis for acute liver failure is poor because

there is no effective treatment other than liver transplantation. Therefore, it is important to study the mechanisms of ALI and develop targeted interventions to increase patient survival.^{2,3}

Oxidative stress results when the amount of reactive oxygen species (ROS) produced in the body exceeds the amount of ROS eliminated by the antioxidant protection mechanism.⁴ ROS can destroy unsaturated fatty acids in the phospholipids of biofilms. A series of cell peroxidation reactions result in the formation of oxidation products that further act on nucleic acids and proteins to destroy the normal functions of cells, resulting in varying degrees of oxidative damage.⁵ Previous research has shown that oxidative stress is involved in all aspects of bodily systems, and diseases such as allergies, asthma, Alzheimer's, diabetes, hypertension, hyperlipidemia, coronary heart disease, and cancer are closely related to oxidative damage.⁶⁻⁸

In addition, a range of cytokines, such as Nrf2, NF- κ B, and p38 kinase, are also activated in the process of anti-oxidative stress. Among them, Nrf2 is one of the core regulators of the endogenous antioxidant system in cells. The lack or activation of Nrf2 will aggravate the cytotoxicity of oxidative stress, thus inducing liver cell apoptosis and inflammatory response, which results in liver injury. Therefore, the Nrf2 signaling pathway is an important target for the treatment of oxidative stress-induced liver injury as the main pathogenesis.⁹ The signaling pathway of Keap1-Nrf2/ARE is considered to be a key pathway of antioxidant stress in vivo. Antioxidant enzymes and Phase II detoxification enzymes regulated in this way can remove ROS and other harmful substances, thus providing detoxification and neutralization.¹⁰

In animal models, D-galactose (D-Gal) is commonly used to induce liver damage. Ingestion of excessive D-Gal that cannot be metabolized will lead to an imbalance in cell function, changes in oxidase activity, and increased production of peroxides and oxidation products, thus causing damage to the structure and function of biomolecules that can cause systemic inflammatory response syndrome, and subsequently lead to acute liver injury and multi-organ failure.¹¹ Lipopolysaccharide (LPS) influences a variety of biological activities and is released after bacteria grow, multiply, die, break, or lyse. By inducing nonspecific immunity in vivo, it can cause inflammatory cytokines to be synthesized and released, which will damage the host's main systems and organs.¹² Liver injury induced by simultaneous D-Gal and LPS injection has been applied to study the pathogenesis of ALI.^{13,14} D-Gal can increase the

sensitivity and thus the lethality of LPS in mice. Although hepatocytes are primarily targeted and produce inflammatory response cells such as macrophages and neutrophils, the mechanism whereby liver damage is induced by D-Gal/LPS is still not fully understood.¹⁵

Antioxidants directly scavenge free radicals, and also increase the activity of antioxidant enzymes, reduce the accumulation of peroxides such as malondialdehyde (MDA) and lipofuscin (LP), and play a protective role.¹⁶ Studies on the antioxidant functions of *Lactobacillus* in vivo and in vitro, for example, *Lactobacillus rhamnosus*, *Lactobacillus fermentans*, and *Lactobacillus plantarum*, indicate that antioxidants are produced during the activities of lactobacillus cells and acellular extracts such as in scavenging free radicals, inhibiting lipid peroxidation, and chelating metal ions.¹⁷⁻¹⁹ The regulating effect of *Lactobacillus* on oxidative damage to the body or cells is reflected by increased expression of antioxidant genes and decreased activity of peroxides in vivo.²⁰

A novel *Lactobacillus plantarum* KSFY06 (LP-KSFY06) was obtained from traditional fermented yoghurt in Kashgar, Xinjiang, China by traditional culture purification. In the current study, D-GalN/LPS-induced ALI model mice were used to study the effect of in vivo *lactobacillus* prophylaxis, and the mechanism of action was explained, thus providing useful information for the development of functional products and the promotion of human health.

Materials and Methods

Strain and Reagents

A new *Lactobacillus* (LAB) strain, *Lactobacillus plantarum* KSFY06 (LP-KSFY06), was registered and stored (preservation number: 15,659) at the China General Microbiological Culture Collection Center (CGMCC, Beijing, China). Lipopolysaccharides (from *Escherichia coli* 055:B5) were obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). D-Galactose was obtained from Sinopharma Chemical Reagent Co., Ltd. (Beijing, China).

Animals

Forty specific-pathogen-free male Kunming mice, 6-8 weeks old, were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). The mice were raised in standard cages in the laboratory animal room (barrier environment) with room

temperature at 20–25°C and relative humidity at 50%-60%. The lights were turned on every 12 hours for illumination, with food and water offered ad libitum.

Cultivation and Treatment of LP-KSFY06

LP-KSFY06 single colonies were inoculated into Man, Rogosa, and Sharpe (MRS, BD, Franklin Lakes, NJ, USA) media contained in liquid culture tubes, cultured at 37°C for 20 h to the initial stage of stabilization, and transferred to MRS liquid medium for 18 h according to the inoculation amount of 3% by volume. The LP-KSFY06 bacteria were collected by centrifugation (4°C, 4000 rpm, 10 min), washed twice with physiological saline, and resuspended to the adjusted cell concentrations of 2.5×10^9 CFU/kg·bw and 2.5×10^{10} CFU/kg·bw.

Antioxidant Experiments in vitro

To determine the antioxidant capability, 0.5 mL of LP-KSFY06 diluent (low concentration: 2.5×10^9 CFU/mL, high concentration: 2.5×10^{10} CFU/mL) was mixed with 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sinopharm Chemical Reagent Co., Ltd., Beijing, China) working liquid (0.33 mmol/L, 2 mL). The absorbance (517 nm) was measured after reaction at room temperature in the dark for 30 minutes (BioMate 3S; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The positive control was 0.2 mg/mL vitamin C (V_C), and there were three parallels in each group.²¹

Next, 0.4 mL of LP-KSFY06 diluent (low concentration: 2.5×10^9 CFU/mL, high concentration: 2.5×10^{10} CFU/mL) was mixed with 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid] (ABTS, Sinopharm Chemical Reagent Co., Ltd., Beijing, China) working liquid (1.0 mL), reacted at room temperature in the dark for 10 min, and then the absorbance (734 nm) was measured. The positive control was 0.2 mg/mL V_C , and these experimental procedures were repeated three times. For the ABTS working solution preparation, reagent A consisted of 3.0 mg of ABTS mixed with 0.8 mL of double-distilled water; reagent B consisted of 1.0 mg of potassium persulfate mixed with 1.5 mL of double-distilled water; reagents A and B were mixed using 0.2 mL of each, oxidized in dark for 12 h, and diluted with anhydrous ethanol to the OD value of A_{734} in the range of 0.7 ± 0.02 .²²

Induction of ALI in Mice

The experimental mice were divided into four groups (normal, model, low dose, high dose) with ten mice/group to study the effect of LP-KSFY06 on D-Gal/LPS-induced liver injury in mice after adaptation (1 week). All

groups were fed a normal diet and water for 4 weeks. The normal group received no additional treatment. The model group received D-Gal (250 mg/kg·bw) and LPS (25 mg/kg·bw) once injected intraperitoneally on the last day of the fourth week. The low- and high-dose groups received daily oral administration of LP-KSFY06 (10^9 CFU/kg·bw for low-dose group, 10^{10} CFU/kg·bw for high-dose group) from weeks two through four and one intraperitoneal injection of D-Gal (250 mg/kg·bw) and LPS (25 mg/kg·bw) on the last day of the fourth week. The volume of injection or oral administration was 0.1 mL/10 g·bw. The experimental design is shown in Figure 1.

Collection of Samples

Two hours after the last gavage, the model group and LP-KSFY06 group were euthanized by 18-hour fasting after intraperitoneal injection of D-Gal/LPS. After taking blood samples, the organs were separated and weighed. Sections (10% formalin) were made of parts of tissues, and the remaining tissues were preserved at -80°C for later experiments. This study was approved by the Ethics Committee of Chongqing Collaborative Innovation Center for Functional Food (201904045B), Chongqing, China and followed the national standard of the People's Republic of China (GB/T 35,892–2018) laboratory animal guidelines for ethical review of animal welfare.

Histological Observations

The liver and spleen tissues (0.5 cm^2) were fixed in 10% neutral formaldehyde for 48 h and then embedded in paraffin and sectioned ($4 \mu\text{m}$). Hematoxylin & eosin staining was used to observe the pathological changes under a BX43 light microscope (Olympus, Tokyo, Japan).

Determination of Biochemical Indicators in Serum and Liver

Mouse blood was placed at 4°C for 2 hours, centrifuged at 3000 rpm and 4°C for 15 minutes (BY-80C centrifugal separator, Beijing Baiyang Medical Instrument Co., Ltd., Beijing, China), and then, the upper serum was obtained and frozen at -80°C . After 0.1 g of mouse liver tissue was added to 0.9 mL of physiological saline, the tissue was homogenized (Bioprep-24; Hangzhou Aosheng Instrument Co., Ltd., Hangzhou, China) three times at a rate of 6 m/s for 30 seconds. Kits were used (Nanjing Jiancheng Institute of Bioengineering, Nanjing, Jiangsu, China) to determine the amounts of aspartate aminotransaminase

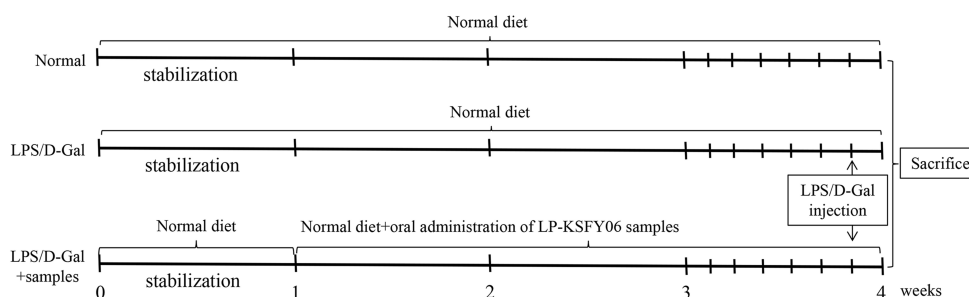


Figure 1 Experimental schedules of a D-Gal/LPS-induced hepatic injury in Kunming mice. Normal group: no additional treatment; Model group: intraperitoneal injection of D-Gal (250 mg/kg bw) + LPS (25 mg/kg bw); Low-dose group: oral administration of LP-KSFY06 (10^9 CFU/kg bw), intraperitoneal injection of D-Gal (250 mg/kg bw) + LPS (25 mg/kg bw); High-dose group: oral administration of LP-KSFY06 (10^{10} CFU/kg bw), intraperitoneal injection of D-Gal (250 mg/kg bw) + LPS (25 mg/kg bw). Injection or oral administration: 0.1 mL/10 g bw. Normal diet composition (mass%): wheat flour (25%), oatmeal (25%), corn flour (25%), soybean flour (10%), fish meal (8%), hog bone powder (4%), yeast powder (2%) and refined salt (1%).

(AST), alanine aminotransaminase (ALT), myeloperoxidase (MPO), MDA, nitric oxide (NO), total antioxidant capacity (T-AOC), catalase (CAT), glutathione (GSH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) in mouse serum and liver.

Determination of Cytokines in the Serum and Liver

The levels of cytokines tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), and interleukin (IL)-1 β , IL-6, and IL-10 in serum and liver were determined by using enzyme-linked immunosorbent assay kits (Abcam, Cambridge, MA, USA).

RT-qPCR Analysis

Liver tissue was homogenized (Bioprep-24, All for Life Science, Co., Ltd., Hangzhou, China), TRIzolTM reagent (Thermo Fisher Scientific) was used to extract the total RNA in the liver, and the concentration was diluted to 1 μ g/ μ L. Total RNA was reverse transcribed into cDNA. Then, 1.0 μ L cDNA, 2.0 μ L forward and reverse primers (10 μ m), and 10.0 μ L premix were mixed to react under the following conditions: 3 minutes at 95°C, 30 seconds at 60°C, and 1 minute at 95°C for 40 cycles. β -Actin was used as the internal reference gene, and the data were calculated by the $2^{-\Delta\Delta CT}$ method.²³ The primer sequences used are shown in Table 1.

Statistical Analysis

All the experimental data were analyzed by SPSS 20.0 software (SPSS Inc, Chicago, IL, USA), combined with one-way ANOVA and Duncan's multiple range tests; $P < 0.05$ was considered to be statistically significant. All the experimental data were obtained in triplicate, and the data are expressed as the mean \pm standard deviation (SD).

Results

Antioxidant Effects of KSFY06 in vitro

As shown in Figure 2, compared to the V_C (ascorbic acid) group (28.39% and 32.26%), the scavenging rate of KSFY06 (2.5×10^9 CFU/mL and 2.5×10^{10} CFU/mL) was 19.91%-48.49% and 38.46%-59.62% for the ABTS and DPPH free radical, respectively ($p < 0.05$). The free radical scavenging rate of KSFY06 was significantly enhanced with increasing concentration, showing a dose-dependent relationship and satisfactory antioxidant properties.

Body Weight and Organ Index of Mice

The organ index indirectly reflects the change in organ function. The weights of the mice in each group were similar (Figure 3), there was no significant difference ($p < 0.05$), and none of them died. As shown in Table 2, compared with the normal group, the indexes of the heart, liver, spleen, lung, and kidney in the model groups were significantly increased due to the stimulation of D-Gal/LPS ($p < 0.05$). Compared with the model group, the organ indexes of the low-dose group and high-dose group decreased after *Lactobacillus plantarum* KSFY06 treatment, and the organ index of the high-dose group decreased more significantly after treatment ($p < 0.05$), indicating that the treatment of *Lactobacillus plantarum* KSFY06 decreased the organ hypertrophy of mice and also reduced the organ damage induced by D-Gal/LPS to a certain extent.²⁴

Histological Analyses

Harmful chemicals are metabolized in the liver, and the morphology of mouse liver tissue with injury induced by D-Gal/LPS will also change.²⁵ As shown in Figure 4, the liver tissue of the normal group of mice was intact, the cell

Table 1 Sequences of Primers Used for RT-qPCR Analysis

Gene Name	Sequence
SOD1	F: 5'-AACCAGTTGTGTTGTGAGGAC-3'
	R: 5'-CCACCATGTTTCTTAGAGTGAGG-3'
SOD2	F: 5'-CAGACCTGCCTTACGACTATGG-3'
	R: 5'-CTCGGTGGCGTTGAGATTGTT-3'
CAT	F: 5'-GGAGGCGGGAACCCAATAG-3'
	R: 5'-GTGTGCCATCTCGTCAGTGAA-3'
GSH	F: TATCAGAGGCGGAAATCTCTT-3'
	R: ATTCTTGCTTCGGCCACATAC-3'
GSH-Px	F: 5'-GTCGGTGTATGCCTTCTCGG-3'
	R: 5'-AGAGAGACGCGACATTCTCAAT-3'
IL-10	F: 5'-CTTACTGACTGGCATGAGGATCA-3'
	R: 5'-GCAGCTTAGGAGCATGTGG-3'
IL-18	F: 5'-GACTCTTGCCTCAACTCAAGG-3'
	R: 5'-CAGGCTGCTTTTGTCAACGA-3'
Trx	F: 5'-TGCTACGTGGTGTGGACCTTGC-3'
	R: 5'-ACCGGAGAACTCCCCACCT-3'
IL-6	F: 5'-CTGCAAGAGACTTCCATCCAG-3'
	R: 5'-AGTGGTATAGACAGGTCTGTTGG-3'
TNF- α	F: 5'-CAGGCGTGCCTATGTCTC-3'
	R: 5'-CGATCACCCGAAGTTCAGTAG-3'
p38	F: 5'-CTGACCGACGACCAGTTC-3'
	R: 5'-CTTCGTTACAGCTAGGTTGC-3'
IFN- γ	F: 5'-GGCCTAGCTCTGAGACAATGAAC-3'
	R: 5'-TGACCTCAAAGTTGGCAATACTC-3'
Keap1	F: 5'-CGGGGACGCGAGTGATGATG-3'
	R: 5'-TGTTAGCTGAAGGTTTCGGTTA-3'
Nrf2	F: 5'-TAGATGACCATGAGTCGCTTGC-3'
	R: 5'-GCCAACTTGCTCCATGTCC-3'
HO-1	F: 5'-GATAGAGCGCAACAAGCAGAA-3'
	R: 5'-CAGTGAGGCCCATACCAGAAG-3'
NQO1	F: 5'-AGGATGGGAGGTAAGTCAATC-3'
	R: 5'-TGCTAGAGATGACTCGGAAGG-3'
NLRP 3	F: 5'-ATTACCCGCCGAGAAAGG-3'
	R: 5'-CATGAGTGTGGCTAGATCCAAG-3'

(Continued)

Table 1 (Continued).

Gene Name	Sequence
ASC	F: 5'-GACAGTGCAACTGCGAGAAG-3'
	R: 5'-CGACTCCAGATAGTAGCTGACAA-3'
IL-1 β	F: 5'-GAAATGCCACCTTTTGACAGTG-3'
	R: 5'-TGGATGCTCTCATCAGGACAG-3'
I κ B- α	F: 5'-CGCGGATGGCCTCAAGAAGGA-3'
	R: 5'-GCCAAGTGCAGGAACGAGTCT-3'
Caspase-1	F: 5'-TGAAGGTAGCAAGACT-3'
	R: 5'-ATAGTGGGCATCTGGGTC-3'
NF- κ B	F: 5'-ATGGCAGACGATGATCCCTAC-3'
	R: 5'-CGGAATCGAAATCCCCTCTGTT-3'
β -Actin	F: 5'-GAGAAAATCTGGCACCACACT-3'
	R: 5'-GCACAGCCTGGATAGCAACGTA-3'

morphology was normal, the hepatocytes were arranged in order, and the central vein was radial. In contrast, the model group exhibited abnormal liver tissue structure, diffuse infiltration of inflammatory cells, uneven staining, irregular central veins, swollen cells, loose cytoplasm, and some cell necrosis. Compared with the model group, the liver cells of mice in the *Lactobacillus plantarum* KSFY06 high- and low-dose groups were neatly rearranged, the morphology was slightly altered, and the hepatocellular edema, necrosis, and inflammation were all reduced to varying degrees.

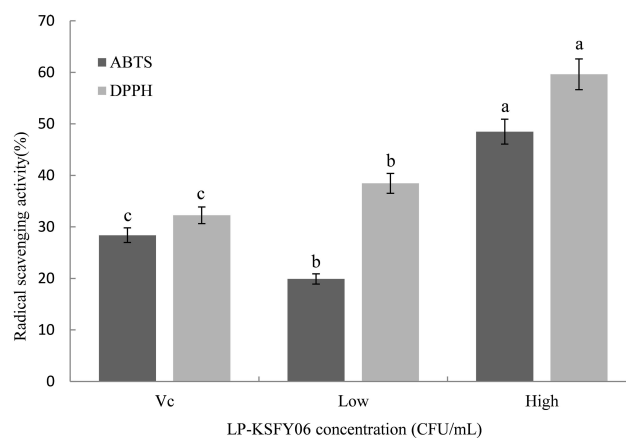


Figure 2 Scavenging activity of KSFY06 towards the DPPH and ABTS free radicals. KSFY06: *Lactobacillus plantarum* KSFY06. Low: mouse treated with 2.5×10^9 CFU/mL KSFY06; High: mouse treated with 2.5×10^{10} CFU/mL KSFY06. ^{a-c}There was significant difference in different letters in the same column ($P < 0.05$), which was determined by Duncan's multiple range test.

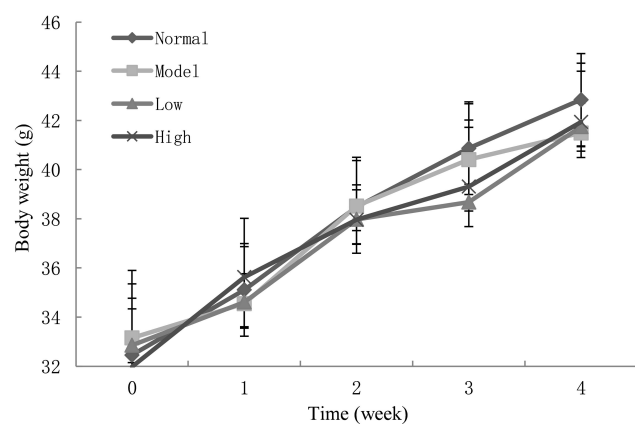


Figure 3 The changes in the weight of mice during the experiment. Low: treated with 2.5×10^9 CFU/mL KSFY06; High: treated with 2.5×10^{10} CFU/mL KSFY06.

In the normal control group, the spleen cells were intact, the tissue structure was clear, the medulla cortex connection was clear, the cells were arranged in an orderly fashion, and no degeneration or necrosis was found (Figure 5). In contrast, model mice exhibited obvious spleen injury, abnormal morphology, structural disorder, enlarged splenic cord, enlarged red pulp sinus, congested spleen, increased red blood cells, decreased white pulp lymphocytes, nuclear fragmentation in white pulp, visibly narrowed red cord, and the arrangement was not dense. In the *Lactobacillus plantarum* KSFY06 groups, the spleen injury was significantly alleviated, the spleen was not congested, there was scattered white pulp in the splenic parenchyma, and the boundary between white pulp and red pulp was clear. Histological observations of the liver and spleen indicated that *Lactobacillus plantarum* KSFY06 may prevent oxidative aging-related tissue damage.

Liver Function-Related Indexes in Serum and Liver Tissue of Mice

ALT and AST are very sensitive to liver injury.²⁶ Through detection, we found that the AST and ALT levels in serum

and liver tissue of the model group significantly increased ($P < 0.05$), with normalization after LP-KSFY06 intervention (Table 3).

SOD, CAT, GSH-Px, GSH, T-AOC, NO, MDA, and MPO Levels in Serum and Liver of Mice

As shown in Figures 6 and 7, the SOD, CAT, GSH-Px, and T-AOC enzymatic activity and GSH content in the model group were lower than those in the normal group, while MPO activity and MDA and NO content were higher than those in the normal group ($p < 0.05$). *Lactobacillus plantarum* KSFY06 treatment effectively inhibited the decrease in serum and liver oxidation-related enzymes and complexes (SOD, CAT, GSH-Px, GSH, and T-AOC) caused by liver injury, and decreased the content of MDA, NO, and MPO, with a stronger effect being observed for the high concentration of *Lactobacillus plantarum* KSFY06. By fully supplementing with exogenous antioxidants (*Lactobacillus plantarum* KSFY06), we can increase the amount and activity of antioxidants in vivo, thus effectively inhibiting free radical oxidation damage, and subsequently preventing various aging diseases caused by oxidative damage.²⁷

Cytokine Levels in Serum and Liver of Mice

As shown in Table 4, the expression of TNF- α , IL-6, IL-1 β , and IFN- γ in serum and liver of the model group was higher than that of the model group, while the expression of IL-10 was lower than that of the model group ($P < 0.05$). In contrast, the *Lactobacillus plantarum* KSFY06 group ameliorated this adverse condition, and the expression of an inflammatory response in mice was decreased.

Table 2 Effects of *Lactobacillus plantarum* KSFY06 on Organ Indices in Mice with D-Gal/LPS-Induced Organ Injury (N=10/Group)

Groups	Heart Index	Lung Index	Liver Index	Spleen Index	Kidney Index
Normal	5.85 \pm 0.84 ^{bA}	5.49 \pm 1.09 ^d	47.67 \pm 2.19 ^c	3.82 \pm 1.39 ^c	13.50 \pm 1.22 ^b
Model	6.17 \pm 1.19 ^a	8.29 \pm 1.21 ^a	50.73 \pm 2.24 ^a	5.54 \pm 1.58 ^a	15.18 \pm 2.93 ^a
Low	5.12 \pm 0.99 ^c	7.34 \pm 1.09 ^b	48.23 \pm 2.51 ^b	4.26 \pm 1.45 ^b	13.25 \pm 1.29 ^{bc}
High	4.29 \pm 0.92 ^d	6.61 \pm 0.90 ^c	47.04 \pm 2.72 ^d	3.45 \pm 1.38 ^d	11.45 \pm 1.99 ^c

Notes: Model: treated with D-Gal/LPS (250 mg/kg bw and 25 mg/kg bw, last day) injection; Low: treated with D-Gal/LPS (250 mg/kg bw and 25 mg/kg bw, last day) injection after *Lactobacillus plantarum* KSFY06 (2.5×10^9 CFU/kg bw, per day) oral administration; High: treated with D-Gal/LPS (250 mg/kg bw and 25 mg/kg bw, last day) injection after *Lactobacillus plantarum* KSFY06 (2.5×10^{10} CFU/kg bw, per day) oral administration. ^{a-d}There was significant difference in different letters in the same column ($P < 0.05$), which was determined by Duncan's multiple range test. ^AValue presented are the mean \pm standard deviation (SD) of different organ coefficients. Organ index (mg/g) = organ weight (mg)/body weight (g).

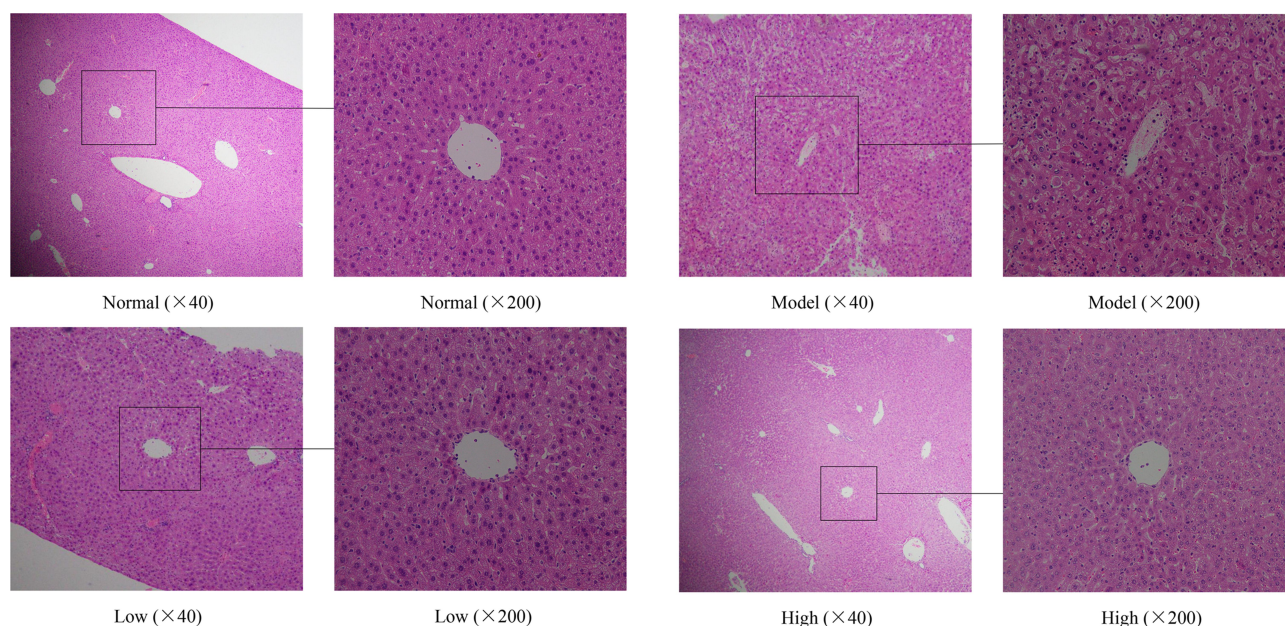


Figure 4 Effects of *Lactobacillus plantarum* KSFY06 on the liver morphology of injured mice. Model: group induced by D-Gal/LPS (250 mg/kg bw, 25 mg/kg bw); Low: treated with LP-KSFY06 (2.5×10^9 CFU/kg bw); High: treated with LP-KSFY06 (2.5×10^{10} CFU/kg bw).

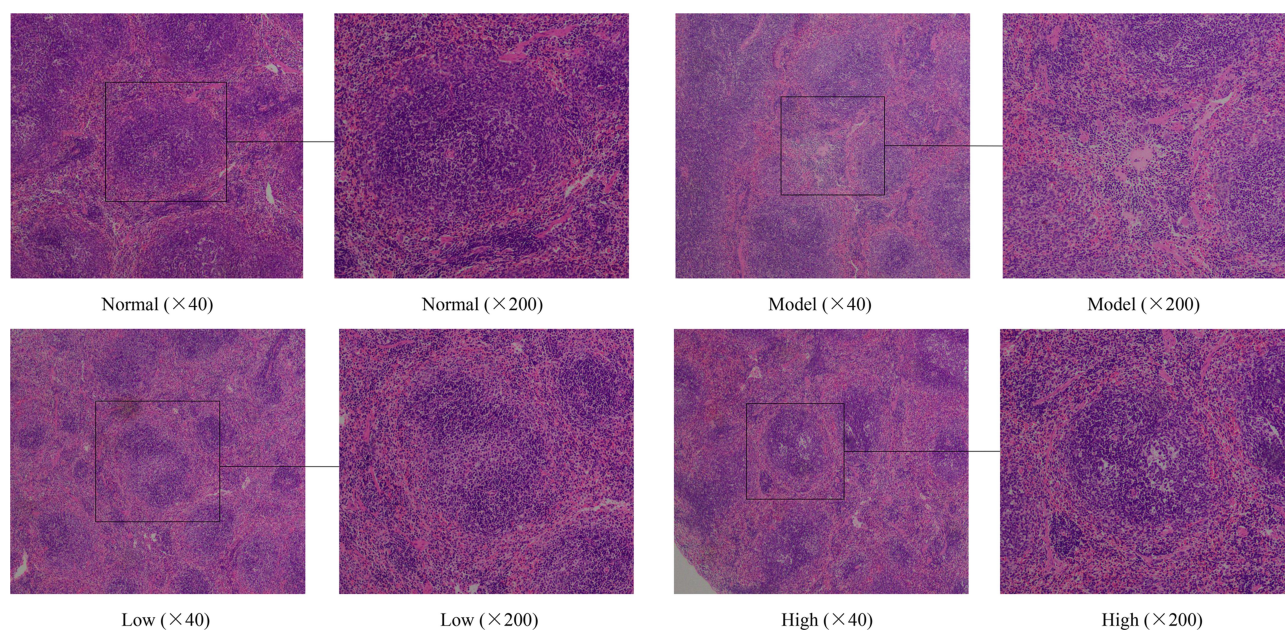


Figure 5 Effects of *Lactobacillus plantarum* KSFY06 on spleen morphology of injured mice. Model: group induced by D-Gal/LPS (250 mg/kg bw, 25 mg/kg bw); Low: treated with LP-KSFY06 (2.5×10^9 CFU/kg bw); High: treated with LP-KSFY06 (2.5×10^{10} CFU/kg bw).

Effects of *Lactobacillus plantarum* KSFY06 on the Gene Expression of Oxidation, Inflammation, and Apoptosis-Related Markers in Mouse Livers (RT-qPCR Assay)

Compared with the normal group (Figure 8), the expression levels of cuprozinc-superoxide dismutase (Cu/Zn-SOD or

SOD1), manganese-superoxide dismutase (Mn-SOD or SOD2), GSH, GSH-Px, CAT, Trx, and IL-10 mRNA in the acute liver injury group (model group) decreased, while IFN- γ , IL-6, IL-18 IL-1 β , TNF- α , and Mapek14 mRNA levels increased, and the expression levels of mRNA after LP-KSFY06 treatment were significantly decreased, indicating that LP-KSFY06 exerted a significant protective effect on

Table 3 Levels of AST and ALT in Serum and Liver Tissue of Mice (N=10/Group)

Groups	AST		ALT	
	Serum (U/L)	Liver (U/g prot)	Serum (U/L)	Liver (U/g prot)
Normal	8.66±1.05 ^{dA}	20.97±1.02 ^c	11.09±1.21 ^c	53.85±1.87 ^c
Model	15.12±1.34 ^a	26.95±1.88 ^a	18.46±1.94 ^a	73.24±1.94 ^a
Low	13.31±1.16 ^b	22.56±1.64 ^b	14.79±1.59 ^b	58.55±1.65 ^b
High	9.69±1.29 ^c	19.93±1.95 ^{cd}	11.03±1.24 ^c	41.18±1.57 ^d

Notes: Model: treated with D-Gal/LPS (250 mg/kg bw and 25 mg/kg bw, last day) injection; Low: mice treated with D-Gal/LPS (250 mg/kg bw and 25 mg/kg bw, last day) injection after *Lactobacillus plantarum* KSFY06 (2.5×10^9 CFU/kg bw, per day) oral administration; High: treated with D-Gal/LPS (250 mg/kg bw and 25 mg/kg bw, last day) injection after *Lactobacillus plantarum* KSFY06 (2.5×10^{10} CFU/kg bw, per day) oral administration. ^{a-d}There was significant difference in different letters in the same column ($P < 0.05$), which was determined by Duncan's multiple range test. ^AValues presented are the mean±standard deviation (SD).

acute liver injury caused by D-Gal/LPS ($P < 0.05$). In addition, the expression of NLRP3, ASC, caspase-1, and NF- κ B mRNA in the model group was higher than that in the normal group, while the expression of I κ B- α mRNA was lower than that in the normal group. The expression of related genes (NLRP3, ASC, caspase-1, and NF- κ B) in the high- and low-dose LP-KSFY06 group was significantly decreased except that the I κ B- α gene was increased ($P < 0.05$), which indicates that the intake of LP-KSFY06 may function through the NLRP3/NF- κ B pathway. Furthermore, the expression of

Keap1 increased, while the expression of Nrf2, HO-1, and NQO1 decreased in the model group (compared to the normal group). After treatment with LP-KSFY06, the expression of Keap1 decreased, resulting in the release and increased expression of nuclear transcription factor Nrf2, activation of downstream antioxidants, and increased expression of HO-1 and NQO1 ($P < 0.05$), which indicates that the intake of LP-KSFY06 through the Keap1-Nrf2/ARE pathway has the effect of inhibiting the oxidative stress injury in mice.

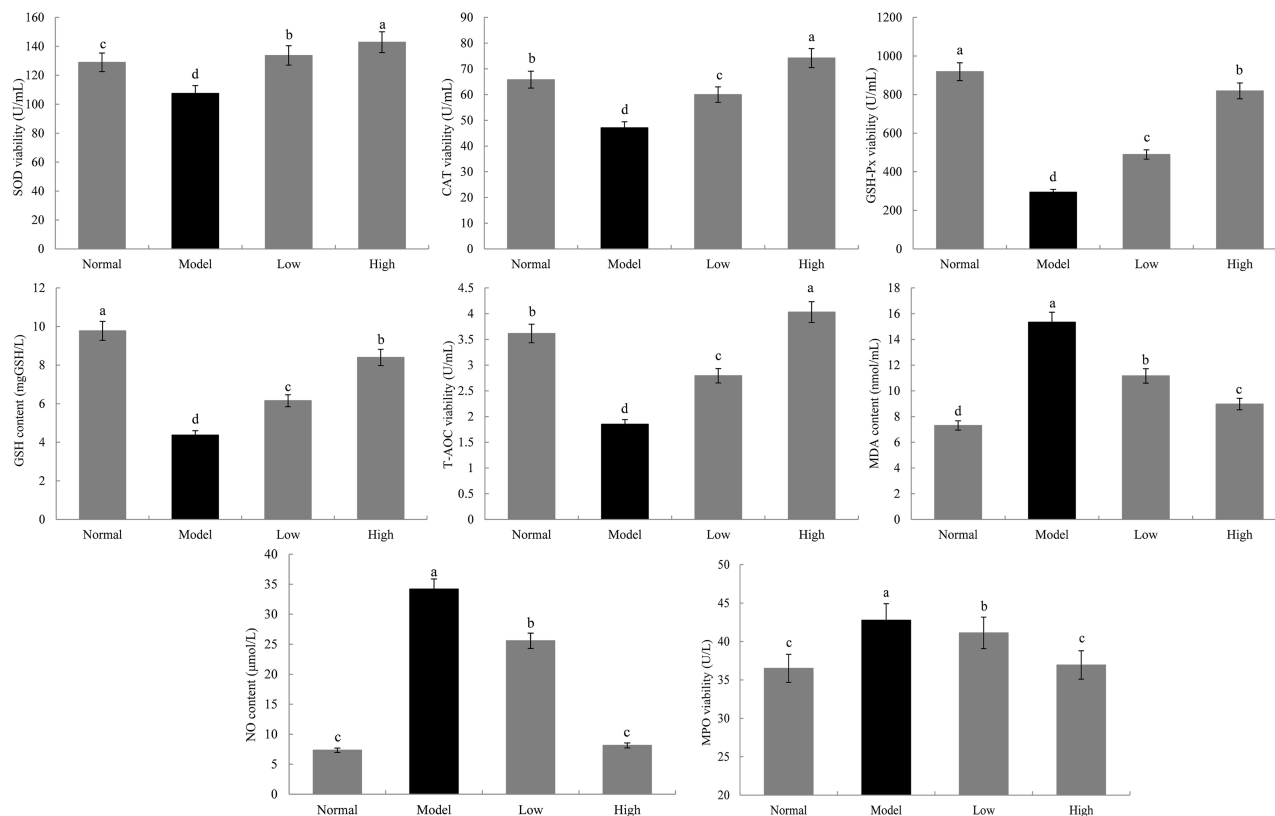


Figure 6 Effects of *Lactobacillus plantarum* KSFY06 on SOD, CAT, GSH-Px, GSH, T-AOC, MPO, MDA, and NO in serum of mice injured by D-Gal/LPS. ^{a-d}There was significant difference in different letters in the same column ($P < 0.05$), which was determined by Duncan's multiple range test. Model: group induced by D-Gal/LPS (250 mg/kg bw and 25 mg/kg bw); Low: treated with LP-KSFY06 (2.5×10^9 CFU/kg bw); High: treated with LP-KSFY06 (2.5×10^{10} CFU/kg bw).

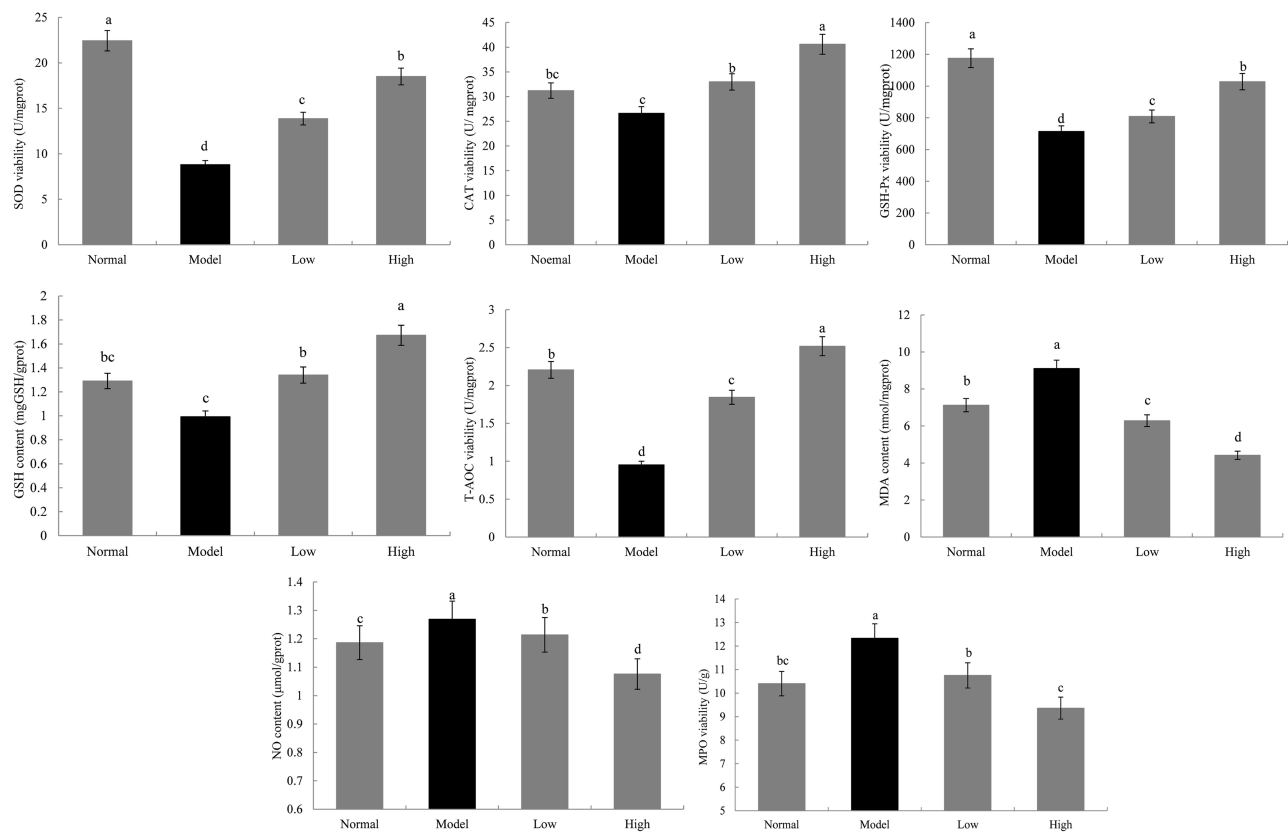


Figure 7 Effects of *Lactobacillus plantarum* KSFY06 on SOD, CAT, GSH-Px, GSH, T-AOC, MPO, MDA, and NO in liver of mice injured by D-Gal/LPS. ^{a-d}There was significant difference in different letters in the same column ($P < 0.05$), which was determined by Duncan's multiple range test. Model: group induced by D-Gal/LPS (250 mg/kg bw and 25 mg/kg bw); Low: treated with LP-KSFY06 (2.5×10^9 CFU/kg bw); High: treated with LP-KSFY06 (2.5×10^{10} CFU/kg bw).

Discussion

Lactobacillus is a probiotic that is widely distributed in nature with species diversity. Some *Lactobacillus* strains

have important biological functions such as anti-oxidation, endotoxin control, anti-aging, anti-cancer, and immunity enhancement.²⁸⁻³² The *Lactobacillus plantarum* KSFY06

Table 4 Levels of IL-6, IL-10, IL-1 β , TNF- α and IFN- γ in Serum and Liver Tissue of Mice (N=10/Group)

Groups	IL-6		IL-10		IL-1 β		TNF- α		IFN- γ	
	Serum (pg/mL)	Liver (pg/mgprot)	Serum (pg/mL)	Liver (pg/mgprot)	Serum (pg/mL)	Liver (pg/mgprot)	Serum (pg/mL)	Liver (pg/mL)	Serum (pg/mL)	Liver (pg/mL)
Normal	5.68 ± 0.71 ^{ba}	4.98 ± 0.47 ^b	58.00 ± 3.83 ^c	176.85 ± 17.49 ^a	21.19 ± 3.35 ^b	12.92 ± 1.34 ^c	61.57 ± 7.32 ^c	84.29 ± 17.37 ^d	86.17 ± 6.76 ^c	80.48 ± 8.37 ^d
Model	8.69 ± 1.34 ^a	6.39 ± 0.54 ^a	45.11 ± 4.76 ^d	124.19 ± 11.96 ^d	26.21 ± 5.08 ^a	16.20 ± 1.62 ^a	97.85 ± 10.38 ^a	119.32 ± 19.4 ^a	110.70 ± 15.41 ^a	112.76 ± 12.34 ^a
Low	5.71 ± 0.49 ^b	4.81 ± 0.37 ^{bc}	60.68 ± 4.38 ^b	137.81 ± 15.94 ^c	19.30 ± 1.84 ^c	15.02 ± 1.05 ^b	82.98 ± 5.71 ^b	107.11 ± 18.13 ^b	90.64 ± 7.37 ^b	100.25 ± 16.22 ^b
High	4.16 ± 1.29 ^c	4.42 ± 0.65 ^c	78.06 ± 7.22 ^a	146.85 ± 19.84 ^b	13.94 ± 2.07 ^d	12.45 ± 2.47 ^{cd}	54.49 ± 6.95 ^c	97.87 ± 11.18 ^c	65.38 ± 6.38 ^c	85.31 ± 13.85 ^c

Notes: Model: treated with D-Gal/LPS (250 mg/kg bw and 25 mg/kg bw, last day) injection; Low: mice treated with D-Gal/LPS (250 mg/kg bw and 25 mg/kg bw, last day) injection after *Lactobacillus plantarum* KSFY06 (2.5×10^9 CFU/kg bw, per day) oral administration; High: treated with D-Gal/LPS (250 mg/kg bw and 25 mg/kg bw, last day) injection after *Lactobacillus plantarum* KSFY06 (2.5×10^{10} CFU/kg bw, per day) oral administration. ^{a-d}There was significant difference in different letters in the same column ($P < 0.05$), which was determined by Duncan's multiple range test. ^AValues presented are the mean \pm standard deviation (SD).

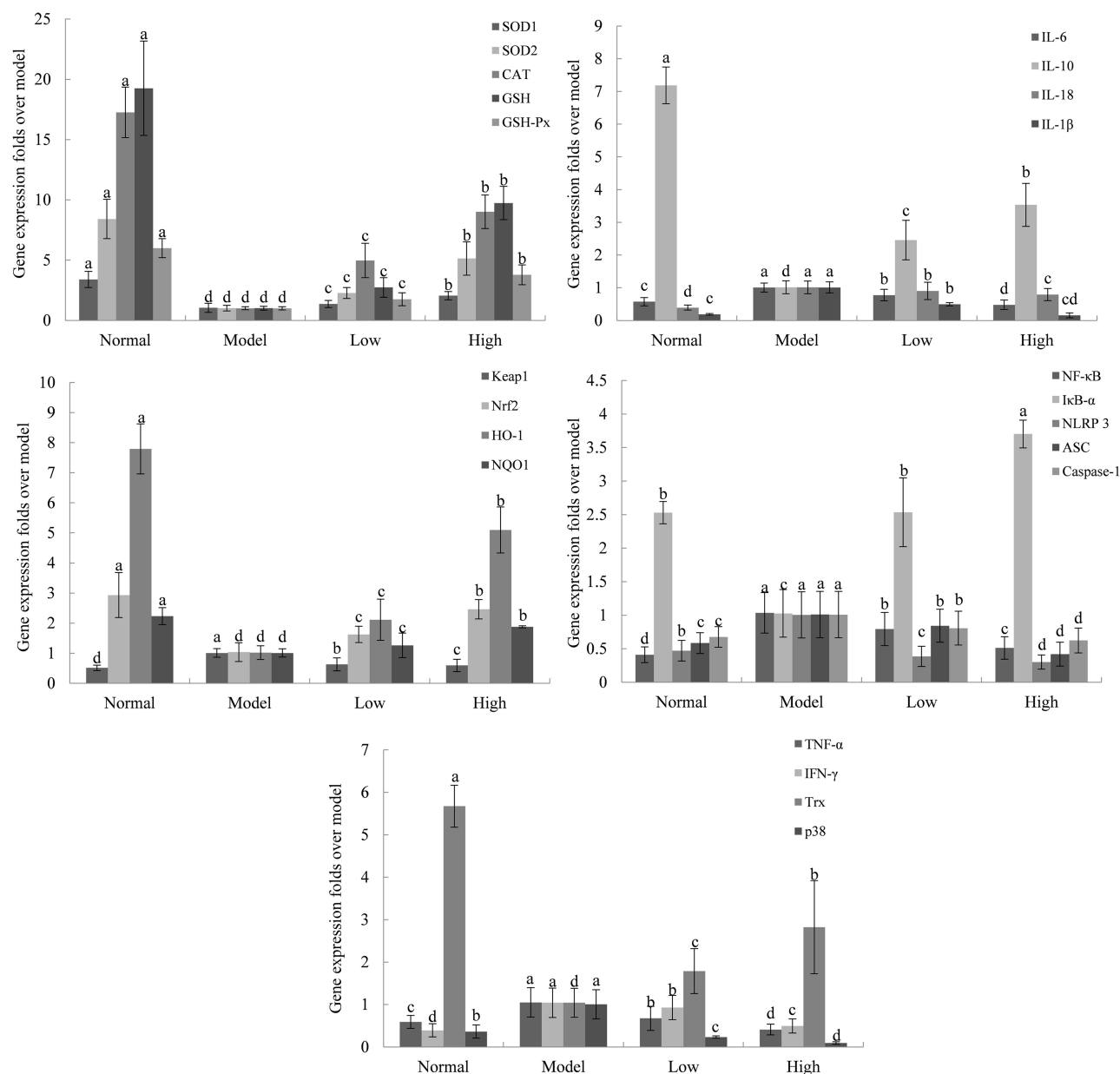


Figure 8 Effects of *Lactobacillus plantarum* KSFY06 on the mRNA expression in liver tissue of mice. ^{a-d}There was significant difference in different letters in the same column ($P < 0.05$), which was determined by Duncan's multiple range test. Model: group induced by D-Gal/LPS (250 mg/kg bw, 25 mg/kg bw); Low: low-dose group, 2.5×10^9 CFU/kg bw; High: high-dose group, 2.5×10^{10} CFU/kg bw.

strain employed in the current study was isolated and identified from naturally fermented milk of cattle produced by the Kashi Herdsman Family in Xinjiang. This high-quality yoghurt was produced in a unique ecological environment with large temperature difference and strong ultraviolet radiation in the Kashi pastoral area, and the separated microorganism also has specific properties that are different from the general microorganism of the same type.³³ Our previous experiments showed that LP-KSFY06 remained active in simulated gastric juice and a bile salt environment.

ALI is a common group of liver syndromes. Viruses, alcohol, drugs, and other factors can cause liver cell swelling, inflammation, and necrosis.³⁴ D-Gal/LPS-induced liver injury models have been widely used in the screening of antioxidants, anti-aging drugs, plant extracts, and probiotics. The mechanism of ALI induced by this model is mainly manifested in oxidative stress and inflammatory response.^{35–38} Long-term injection of galactose in mice resulted in incomplete metabolic functions and accumulation in vivo, with changes in cell osmotic pressure, cell

swelling, metabolic disorder, and ultimately, oxidative stress response to liver injury.³⁹ LPS is an endotoxin that is mainly eliminated by Kupffer cells, which are activated in endotoxemia and produce ROS such as hydrogen peroxide to cause oxidative stress, and also secrete inflammatory factors such as IL and TNF- α , leading to cell necrosis.⁴⁰ In our study, we found that D-Gal/LPS induced severe hepatocyte necrosis, inflammatory cell production (lymphocytes and macrophages), and disorder of liver cells around the central vein. However, after LP-KSFY06 treatment, the pathological degree of liver injury was significantly decreased, which had a positive preventive effect on liver injury.

Organ weight and organ index reflect the damage status of the body to a certain extent. LP-KSFY06 can prevent the increase in the organ index induced by D-Gal/LPS.⁴¹ ALT is the most sensitive in the early stage of ALI, and it is the main index used to diagnose the damage to the hepatocyte parenchyma, whereas AST is the main index that reflects the degree of liver injury.⁴² The presence of transaminase in serum and liver tissue indicated that LP-KSFY06 inhibited ALT and AST from increasing. SOD catalyzes the superoxide anion free radical dismutation to produce oxygen and hydrogen peroxide through the alternative electron gain and loss of metal ions M^{n+1} (oxidation state) and M^n (reduction state), which plays a role in the oxidation balance of the body.⁴³ CAT catalyzes the decomposition of H_2O_2 into H_2O and O_2 , protects cells from H_2O_2 , and interacts with SOD.⁴⁴ GSH is a major nonenzymatic scavenger that regulates redox homeostasis in cells.⁴⁵ GSH-Px can catalyze GSH to produce GSSG, and they work together to inhibit the systemic edema caused by oxidative stress, thus delaying the oxidative aging of the body.⁴⁶ In our study, antioxidant enzymes (SOD, CAT, and GSH-Px) and nonenzymatic antioxidants (GSH) interacted with LP-KSFY06 to act as antioxidants.

Free radicals act on lipids to produce peroxidation reactions, and the end product of oxidation is MDA, which will cause cross-linking polymerization of proteins, nucleic acids, and other macromolecules, and has cytotoxicity, thus affecting the biochemical reactions of normal tissues.⁴⁷ NO is fat soluble, rapidly diffuses through biofilm, and plays an important role in the regulation of heart and cerebral vessels.⁴⁸ MPO is derived from polymorphonuclear neutrophils (PMNs), monocytes, and macrophages, which are stored in azurophilic granules. When leukocytes are activated and degranulated, MPO is released, and this occurs in response to immune defense

and tissue damage in many diseases such as leukemia, vasculitis, and Alzheimer's disease.⁴⁹ When external stimulation causes oxidative stress damage (D-Gal/LPS), the metabolism of the body is disordered, and the initial balance state cannot be maintained. The lipid peroxidation products (MDA, NO, MPO) were significantly increased, and LP-KSFY06 acted as an antioxidant by reducing the content and activity of these products.

With the aggravation of liver injury, peroxides increase, and simultaneously, oxidative stress injury leads to low immune function and secretion of inflammatory cytokines. Oxidative stress injury can also act on hepatic stellate cell (HSC) and Kupffer cells, forming positive feedback regulation, leading to the increase and aggregation of cytokines, and thus promoting the development of liver injury.⁵⁰ IL-1 β , IL-6, IL-10, TNF- α , and IFN- γ in serum are the most important immunoregulatory cytokines in the body. The function of the immune system is related to Th1 or Th2 cells, the secretion of IFN- γ is related to Th1 cells, and the secretion of IL-6 and IL-10 is related to Th2 cells.⁵¹ TNF- α and IL-6 can act on the receptors located on the surface of hepatocytes, and they are clearly toxic to the liver and cause necrosis of a large number of hepatocytes. TNF- α is the earliest and most important mediator in the inflammation process, and IL-6 promotes the accumulation of acute proteins and T cells at the inflammation site.^{52,53} IL-1 β is mainly expressed by innate immune cells in inflammatory injury, and its precursor intracellular processing depends on caspase-1.⁵⁴ IFN- γ inhibits Th2 development, and IL-10 inhibits Th1 reaction, and while these two normally regulate each other, this homeostasis is destroyed in the process of inflammation.⁵⁵ In the current study, LP-KSFY06 downregulated TNF- α , IFN- γ , IL-6, and IL-1 β , and upregulated IL-10, thereby reducing the expression of stress factors in injured mice.

The Keap1-Nrf2/ARE pathway is considered to be the most important endogenous antioxidant signaling pathway. Under normal physiological conditions, Nrf2 mainly binds to its inhibitor Keap1, exists in the cytoplasm in its inactive state, and is rapidly degraded under the action of the ubiquitin proteasome pathway to maintain the low transcriptional activity of Nrf2 under physiological conditions. D-Gal/LPS enhanced the expression of negative regulator Keap1 and inhibited the expression of Nrf2, HO-1, and NQO1. LP-KSFY06 ingestion causes Nrf2/ARE signal activation, lower relative expression of Keap1, release of nuclear transcription factor Nrf2,

enhanced binding of Nrf2 and ARE, and further activation of downstream antioxidant enzymes and type II detoxification enzyme genes, and the body participates by regulating antioxidant activity. Nrf2 is an important transcription factor that regulates the oxidative stress response of cells, which can reduce the cell damage caused by ROS and electrophilics, maintain cellular stability, and maintain the dynamic balance of redox by inducing and regulating the expression of a series of antioxidant proteins. HO-1, as a stress protein, plays a role in hemoglobin metabolism, inflammation, and the antioxidant process, and also has a protective effect on the cardiovascular and nervous systems.^{56–58}

NF- κ B belongs to the Rel protein family, and the heterodimer composed of p65/P50 is the main biologically active form, which combines with I κ B- α to form a trimer (inactive form). D-Gal/LPS can activate a series of protein kinases, such as activation of the I κ B kinase complex, which leads to I κ B- α ubiquitination degradation and the production of NLRP3 inflammatory bodies, and then mediates an inflammatory response. NLRP3 inflammatory corpuscles can lead to the self-shearing of the inflammatory aspartate-specific cysteine proteolytic enzyme, which initiates the host's inflammatory response by activating proinflammatory factors such as IL-1 β .^{59–61} Previous studies have documented that the NF- κ B/NLRP3 pathway plays an inflammatory role in the process of ALI.⁶² We found that LP-KSFY-6 treatment enhanced the expression of I κ B- α , which inhibited the expression of NF- κ B, NLRP3, ASC, and caspase-1 in the NF- κ B/NLRP3 pathway.

In addition, we demonstrated the anti-oxidant and anti-inflammatory effects of LP-KSFY06 in molecular biology, histology and at the gene level. We have not examined these effects at the protein level, but we plan to do so in future experiments.

Conclusion

LP-KSFY06 ameliorated the ALI induced by D-Gal/LPS, increased the activity of antioxidant enzymes in the liver and serum of injured mice, reduced the amount of peroxides, and increased the level of immunoregulatory factors. Its mechanism is related to increasing the body's antioxidant capacity and enhancing the body's immunoregulatory function. LP-KSFY06 activated the Nrf2/ARE signaling pathway, and inhibition of the NF- κ B/NLRP3 signaling pathway plays a role.

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Disclosure

The authors report no conflicts of interest for this work.

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