Preventive Effect of Lactobacillus Plantarum HFY15 on Oxidative Damage and Acute Liver Injury Induced by Carbon Tetrachloride (CCl₄) in Mice

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Abstract

Background

Carbon tetrachloride (CCl₄) is a widely used hepatic toxin that causes acute liver injury through pathological mechanisms such as oxidative stress, inflammation, and apoptosis. In this experiment, mice were treated with *Lactobacillus plantarum* (LP) HFY15, silymarin, and *Lactobacillus delbruechii* subsp. *bulgaricus* (LDSB) for two weeks, and intraperitoneal injection of CCl₄-induced acute liver injury to study the preventive effect of LP-HFY on CCl₄-induced acute liver injury, especially in oxidative damage.

Results

The survival rate of LP-HFY15 in artificial gastric juice is 92.1%, and the growth efficiency in bile salt is 78.8%. Animal experiments show that LP-HFY15 reduces the liver index of mice, reduces the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), the content of triglycerides (TG), malondialdehyde (MDA) and reactive oxygen species (ROS) in the serum of mice. LP-HFY15 also increased the antioxidant genes, such as *nuclear factor erythroid 2-related factor 2* (*Nrf2*), *heme oxygenase* (*HO-1*), *NAD(P)H:quinone oxidoreductase* (*NQO1*), and increases the activity of *superoxide dismutation* (*SOD*), *catalase* (*CAT*), and *glutathione* (*GSH*) in the serum and gene level. At the same time, LP-HFY15 reduced the levels of cytokines IL-6, TNF- α , and IFN- γ in the serum and liver, increased antiapoptosis gene *B-cell lymphoma-2* (*Bcl-2*) expression in the liver of mice, and inhibit the expression of proapoptotic genes *Bcl-2-associated X* (*Bax*) and *Caspase-3*.

Conclusions

Compared with LDSB, LP-HFY15 has a greater alleviating effect on the liver injury caused by CCl₄. These findings demonstrate that LP-HFY15 has great potential and research value in the future as a food supplement for preventing acute liver damage.

Background

The liver has multiple functions such as detoxification, metabolism, and synthesis, which play an important role in maintaining health. At the same time, the liver is also vulnerable to damage. Drugs, viruses, alcohol, and other harmful substances may cause liver inflammation, necrosis, fibrosis, cirrhosis, and eventually liver cancer [1–3]. Various liver diseases are usually caused by acute liver damage caused by harmful substances [4]. The pathogenesis of acute liver injury is known to involve the interaction of processes such as apoptosis, oxidative stress, and inflammation [5]. Although many drugs can be used to treat liver damage, drug treatment has side effects and can also cause damage to the body. Therefore, it is essential to find new ways to prevent liver damage.

Probiotics refer to dietary supplements containing live microbial strains, which amplify the beneficial effects of the normal intestinal flora by exogenous microorganisms from food to improve the health of

the host [6]. Lactic acid bacteria is a microbial species widely used as probiotics in the food industry. It has been proven to have multiple functions such as regulating skin health [7], reducing memory impairment in mice [8], and inhibiting enterovirus Coxsackie virus B4 (CV-B4) [9]. Other studies have shown that *Lactobacillus fermentum* LA12 can be used to prevent and treat alcoholic liver injury [10]; *Lactobacillus plantarum* (LP) C88 can prevent liver injury in mice induced by aflatoxin B1 [11]; and LP-DSMZ 2017 protects the liver through antioxidant effects [12]. The protective function of lactic acid bacteria as a functional food on the liver has been confirmed. The main mechanisms of carbon tetrachloride (CCl₄)-induced acute liver injury in mice are oxidative stress, inflammation, and apoptosis [13]. This is similar to human acute chemical liver injury, so it is widely used in animal models to find potential treatment strategies [14]. However, oxidative stress is the main pathogenic factor of liver injury induced by CCl₄. In the process of liver metabolism of CCl₄, hepatotoxic metabolites and excessive free radicals are produced, which deplete reducing agents such as GSH, inhibit the production of antioxidant enzymes such as SOD and CAT, and induce oxidative stress. At the same time, toxic metabolites and free radicals combine with phospholipid molecules, leading to lipid peroxidation and membrane dysfunction. In addition, they can also bind to other macromolecules, such as proteins and DNA, which can cause cell damage [15].

Yak yogurt is a fermented milk, which is commonly found in ethnic minority areas on the plateau of China. It contains more protein, essential amino acids, and fats than ordinary milk, so it has extremely high nutritional value [16]. Owing to the unique climate, altitude, and technical conditions of the plateau, the taste of yak yogurt and its microbial community are also very rich [17]. The health function of yak yogurt is closely related to the dominant lactic acid bacteria present in it [18]. Experiments have shown that the dominant genus of bacteria in yak milk is *Lactobacillus*. Preliminary laboratory studies have shown that LP-HFY05 isolated from natural fermented yak milk can reduce alcoholic liver injury in mice [19]; LP-KSFY02 can prevent d-galactose-induced oxidative senescence in mice [20]; and LP-ZS62 can reduce alcoholic gastric injury in mice through antioxidant mechanism [21].

In order to study the preventive effect of *L. plantarum* on CCl₄-induced acute liver injury, LP-HFY15 isolated from yak yogurt sampled in the Hongyuan area of Sichuan province was used to gavage mice in this study. By measuring the corresponding indicators of mouse liver and serum, combined with liver histopathological slices, the degree of prevention of LP-HFY15 on the acute liver injury caused by CCl₄ and its possible mitigation mechanism were explored.

Results

Isolation and identification of *L. plantarum* HFY15

The colony is white, round, translucent, with a smooth and matt surface, and a raised center and neat edges (Fig. 1A). Gram staining showed Gram-positive bacteria, which were mainly short and elongated under the microscope, without branches (Fig. 1B).

Tolerance of artificial gastric juice and bile salts

Resistance to gastric juice and bile salts in the gastrointestinal tract is a prerequisite for the function of probiotics [24]. Therefore, the survival rate of LP-HFY15 under artificial gastric juice and *in vitro* growth efficiency under bile salt treatment were determined. As shown in Fig. 2, the survival rate of LP-HFY15 in artificial gastric juice at pH 3.0 is greater than 90%, and it can maintain good growth activity in 0.3% bile salts.

Weight and liver indicators

The liver organ index is the ratio of the weight of the liver tissue of the mouse to its body weight. As shown in Table 1, the average liver weight and liver index of the normal group are the lowest. After CCl₄ treatment, the mice had the highest average liver weight and liver index. Treatment with LP-HFY15 reduced the liver weight and liver index. The liver index of silymarin group and LDSB group also had a downward trend compared with CCl₄, but the effect was not as significant as that of LP-HFY15.

Table 1
Mouse body weight, liver weight, and liver index.

	Normal group	CCl ₄ -induced group	Silymarin group	LP-HFY15 group	LDSB
Body weight (g)	35.54 ± 1.50 ^a	27.75 ± 1.90 ^d	32.25 ± 1.64 ^b	33.98 ± 1.81 ^a	29.98 ± 1.50 ^c
Liver weight (mg)	1.53 ± 0.24 ^b	1.99 ± 0.37 ^a	1.62 ± 0.14 ^b	1.61 ± 0.14 ^b	1.74 ± 0.17 ^b
Liver index (%)	4.30 ^d	7.17 ^a	5.02 ^c	4.74 ^d	5.80 ^b
^{a-e} Mean values with different letters in the same column are significantly different (<i>p</i> < 0.05) according to Duncan's multiple range test. CCl ₄ -induced group: mice were injected with 10 mg/kg CCl ₄ on the 14th day; Silymarin group: mice were given 50 mg/kg silymarin every day and injected with 10 mg/kg CCl ₄ on the 14th day; LP-HFY15 group: mice were treated with 1.0×10 ⁹ CFU/kg (b.w.) of <i>L. plantarum</i> HFY15 every day and injected with 10 mg/kg CCl ₄ on the 14th day; LDSB group: mice were treated with 1.0×10 ⁹ CFU/kg (b.w.) of <i>L. delbruechii</i> subsp. <i>bulgaricus</i> every day and injected with 10 mg/kg CCl ₄ on the 14th day.					

Analysis of mouse serum ALT, AST, and TG

The levels of ALT, AST, and TG in mouse serum are shown in Fig. 3. ALT and AST levels are often used as indicators to evaluate liver damage. Compared with the normal group, the levels of ALT, AST, and TG in the CCl₄-induced group significantly increased. Compared with the CCl₄-induced group, the ALT, AST, and

TG levels in the LP-HFY15 group were significantly reduced. Silymarin is widely used in the treatment of liver damage as a positive control. The silymarin group and LDSB group also had the same trend. However, the levels of silymarin group and LP-HFY15 group were close to the normal group, and a significant difference from the LDSB group was observed.

Analysis of MDA, T-SOD, ROS, GSH and CAT in mouse serum

Serum MDA content, SOD, ROS, GSH and CAT activity levels are shown in Fig. 4. MDA, SOD, and CAT are important indicators of oxidation. It could be seen from the figure that MDA and ROS levels in mouse serum in the normal group were the lowest among the five groups, and SOD, GSH and CAT were the highest among the five groups. Compared with the normal group, the MDA content and the ROS level of CCl₄-induced group significantly increased, and the activities of SOD, GSH and CAT significantly decreased. After the treatment with LP-HFY15, the oxidation index trend was opposite to that of CCl₄-induced group, which reduced the content of MDA and the level of ROS, and increased the activity of SOD, GSH and CAT. The silymarin group and the LDSB group showed the same oxidation index trend as the LP-HFY15 group, and the values of the silymarin group and LP-HFY15 group were closer to the normal group.

Analysis of serum IL-6, TNF- α , and IFN- γ levels in mice

The serum cytokine levels are shown in Fig. 5. Serum cytokines are often used as indicators of liver inflammation. The levels of IL-6, TNF- α , and IFN- γ in the CCl₄-induced group were significantly higher than those in the normal group. Compared with the CCl₄-induced group, the LP-HFY15 group, LDSB group, and silymarin group significantly reduced the levels of IL-6, TNF- α , and IFN- γ . The silymarin group was closest to the normal group, followed by the LP-HFY15 group. The expression level of LDSB group was significantly higher than those of silymarin group and LP-HFY15 group.

Histopathological observation

The H&E stained section of the mouse liver is shown in Fig. 6. In the normal group, the liver cells were intact, the nuclei were neatly arranged, and the liver lobules were clearly structured and evenly distributed. The CCl₄-induced group showed severe liver cell degeneration, liver lobule disorder, nuclear condensation, and a large number of inflammatory cell infiltrations. In the silymarin group, LP-HFY15 group, and LDSB group, the liver cell degeneration was reduced, nuclei were arranged more neatly, and inflammatory cells were slightly infiltrated.

qPCR result analysis

The qPCR results are shown in Fig. 7. CCl₄ reduced the expression of genes *CAT*, *SOD1*, *SOD2*, *Bcl-2*, *Nrf2*, *HO-1*, and *NQO1*, and increased the expression of genes *TNF-α*, *IFN-γ*, *IL-6*, *Bax*, and *Caspase-3*. Compared with the CCl₄-induced group, LP-HFY15 can significantly increase the expression of *CAT*, *SOD1*, *SOD2*, *Bcl-2*, *Nrf2*, *HO-1*, and *NQO1*, and reduce the expression of *TNF-α*, *IFN-γ*, *IL-6*, *Bax*, and *Caspase-3* genes. The results of silymarin group and LDSB group are similar to those of LP-HFY15 group, but after silymarin and LP-HFY15 treatment, the related gene expression is closer to the normal group.

Discussion

As a type of edible probiotics, lactic acid bacteria can regulate the imbalance of intestinal microbiota composition by increasing the number of bacteria, improving the intestinal epithelial barrier function, and promoting the production of cytokines to prevent obesity, cancer, liver damage, and other diseases [23]. In recent years, studies have been conducted on the use of lactic acid bacteria to alleviate CCl₄-induced liver injury [2]. Therefore, the LP-HFY15 strain isolated from the yak yogurt in the Hongyuan area of Sichuan Province was used in this study to explore its protective effect on CCl₄-induced liver injury.

Lactic acid bacteria must be able to tolerate the acidic environment of the gastrointestinal tract and then reach the intestine through food or oral intake [24]. The bile salts in the small intestine will destroy the cell membrane of the bacteria. Therefore, the lactic acid bacteria must have a certain ability to resist gastric juice and bile salts. Only with good tolerance can it reach the intestine with a higher number of viable bacteria and colonize the intestine to exert its probiotic effect. The results show that the survival rate of LP-HFY15 artificial gastric juice treatment was 92.1%, and the growth efficiency under 0.3% bile salt was 78.8%. The survival rate of *L. fermentum* Lee used in the experiment at pH 3.0 in artificial gastric juice was 87.99%, and the growth efficiency under 0.3% bile salt was 25.31% [25]. In contrast, LP-HFY15 has good resistance to gastric acid and bile salt.

CCl₄ can cause liver damage and liver fibrosis in mice [26]. Liver histopathology is an important clinical criterion for diagnosing liver injury [27]. Through experiments, it was found that CCl₄ treatment caused obvious swelling of mouse liver cells, irregular nucleus size, and lymphocyte infiltration. After the treatment with LP-HFY15, the condition improved, and the liver cells were slightly swollen. Liver weight and liver indicators are usually used as the indicators of liver injury induced by CCl₄ [28]. The results show that treatment with LP-HFY15 could reduce the liver weight and liver indexes in the CCl₄-induced group, indicating that LP-HFY15 played a positive role in relieving CCl₄-induced liver injury.

ALT and AST are indispensable catalysts in the normal functioning of the liver. ALT exists in liver cells, while AST exists in the mitochondria of liver cells. When liver cells are damaged, ALT will enter the blood first, and when liver cells are severely damaged, ALT will also enter the blood, leading to an increase in the transaminase in the serum of mice [29, 30]. Therefore, ALT and AST levels can be used to evaluate the degree of liver damage, and their values are positively correlated with the degree of liver cell damage [31]. Liver damage can cause fat in peripheral adipose tissue to be transported to the liver and accumulate, increasing the content of TG in the liver [32]. The results show that LP-HFY15 can regulate the levels of

ALT and AST in the liver of mice, reduce the content of TG in the CCl₄-induced group, and reduce the damage of CCl₄ to the liver.

Acute liver injury is related to liver oxidative stress [33]. The damage caused by oxidative stress can be mitigated by the enzymatic antioxidant defense system [34]. Therefore, this study evaluated the liver's oxidative stress ability by measuring SOD, CAT, ROS, GSH, and MDA and other antioxidant parameters. SOD and CAT are antioxidant enzymes. SOD can catalyze the disproportionation reaction of superoxide anions and scavenge free radicals, while CAT can eliminate hydrogen peroxide in the body and enhance the role of SOD in scavenging free radicals [35]. Reduced consumption of endogenous antioxidants can increase the sensitivity of liver cells to oxidative stress. Therefore, SOD, CAT, and GSH are often used to evaluate the antioxidant activity in the body [15]. MDA is the final metabolic product of lipid peroxidation, and it is considered an important marker of oxidative stress [36]. In addition, the defense system of antioxidant enzymes may reduce oxidative stress by reducing ROS [37]. Experiments show that LP-HFY15 can significantly increase the activity of SOD, GSH, and CAT in mice and reduce the level of MDA and ROS, thereby reducing the oxidative damage caused by CCl₄ to the liver.

The Nrf2 pathway is an important body's self-defense system that can protect tissues from oxidative stress. The transcription factor Nrf2 is the main regulator of cellular antioxidant defense response, and is related to the endogenous antioxidant system [38]. Nrf2 generally exists in the cytoplasm in an inactive form, maintaining low levels of Nrf2 to regulate gene expression. Under oxidative stress, Nrf2 transfers from the cytoplasm to the nucleus, activating the transcription of a variety of antioxidant and detoxification genes, which including HO-1 and NQO1 [39]. HO-1 and NQO1 are the downstream antioxidant proteins of Nrf2. HO-1 has anti-inflammatory, anti-apoptotic and anti-oxidant effects on fibroblasts, hepatocytes and renal epithelial cells. HO-1 also can reduce the amount of mitochondrial oxidation products by inducing autophagy, thereby protecting the heart [40, 41]. NQO1 is mainly located in the cytoplasm, but there are also lower levels in the nucleus. Nrf2 has an antioxidant effect by inducing the expression of NQO1 isoenzymes [42, 43]. After evaluating the expression of Nrf2 and its downstream molecules HO-1 and NQO1, we observed that the Nrf2-mediated pathway is inhibited in the CCl₄-induced group. LP-HFY15 shows a protective effect by up-regulating the expression of these molecules, which means LP-HFY15 plays an anti-oxidant effect in CCl₄-induced liver injury by activating the Nrf2-mediated pathway. These results indicate that the protection of cells from CCl₄-induced oxidative stress by LP-HFY15 is related to the regulation of antioxidant enzymes, lipid peroxidation and liver antioxidant gene expression.

Inflammatory cytokines play a key role in liver injury [44]. CCl₄ induces liver oxidation and inflammation, releases various inflammatory mediators during oxidative stress injury, and significantly increases the levels of serum inflammatory factors IL-6, TNF- α , and IFN- γ in mice. [36] IL-6 promotes the proliferation and differentiation of T lymphocytes and enhances the body's inflammatory response [33, 45]. TNF- α can accumulate inflammatory cells, leading to inflammatory cell infiltration and tissue edema [46]. IFN- γ can increase the sensitivity of liver cells to TNF- α and further damage liver cells [47]. It was experimentally

found that LP-HFY15 can significantly downregulate the levels of IL-6, TNF- α , and IFN- γ , indicating that LP-HFY15 has a good alleviating effect on the inflammatory response induced by CCl₄ in mice.

Apoptosis is a form of programmed cell death. It is the main mechanism that regulates cell death. It usually occurs during the development or aging. It also occurs as a defense mechanism when cells are damaged or stressed, and the damaged cells are removed in an orderly and effective manner through multigene control [48, 49]. The mitochondrial pathway regulated by the Bcl-2 family of proapoptotic and antiapoptotic proteins has been shown to play an important role in CCl₄-induced apoptosis [5]. Bcl-2 is mainly located on the membrane of intracellular organelles and has an antiapoptotic effect. Bax protein is mainly distributed in the cytoplasm. When the cell receives an apoptosis signal, Bax will migrate from the cytoplasm to the mitochondrial membrane, causing damage to the mitochondrial membrane and promoting apoptosis [50, 51]. The downregulation of Bcl-2 and the upregulation of Bax will cause mitochondria to release cytochrome c, thereby activating caspase in the cytoplasm, the most important of which is Caspase-3, which ultimately leads to cell apoptosis [52]. According to the experimental data, LP-HFY15 can significantly upregulate the expression of Bcl-2 and downregulate the expression of Bax and Caspase-3, indicating that LP-HFY15 can alleviate the apoptosis of liver cells caused by CCl₄, and protect the normal physiological functions and procedures of liver cells.

Conclusion

In summary, LP-HFY15 has higher resistance to gastric acid and better bile salt survival ability. It can inhibit the production of proinflammatory factors and improve the liver's anti-inflammatory ability. It can also prevent liver cell apoptosis by inhibiting the expression of proapoptotic genes and promoting the expression of antiapoptosis, and ultimately maintaining the normal morphology of liver tissues and liver cells. Moreover, LP-HFY15 can scavenge-free radicals, regulate the release of antioxidant-related enzymes, control liver fat oxidation, and avoid peroxidation. This study provides a basis for the future development of functional foods related to LP-HFY15 to prevent liver damage induced by chemical poisons.

Materials And Methods

Separation of strains

The samples used in this experiment were collected from yak yogurt in Hongyuan, Sichuan, China. Then, 1 mL of yogurt was added to 9 mL of sterile normal saline, mixed well, and then diluted gradually. Next, 100 μ L of bacterial solution with four dilutions of 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} was taken, spread, and inoculated on de Man, Rogosa, and Sharpe (MRS) (288130, Becton, Dickinson and Company, NJ, USA) solid medium, and incubated at 37°C for 24–48 h. The morphology of the colony was observed; a suitable colony culture was selected and streak with MRS medium to isolate the pure strain. The pure colonies were inoculated into 5 mL MRS liquid medium, incubated for 16–18 h at 37°C, 1 mL of culture solution was taken and centrifuged at 12000 rpm for 5 min. The supernatant was discarded, and 500 mL

sterile normal saline was added. The solution was mixed well, and Gram staining was conducted. Finally, a microscopic examination was conducted.

Identification and preservation of strains

LP-HFY15 was identified using the Basic Local Alignment Search Tool (BLAST) in the National Center of Biotechnology Information (NCBI). This strain is currently stored in China General Microbiological Culture Collection Center (CGMCC, Beijing, China); the preservation number of LP-HFY15 is CGMCC No. 16648. Another positive control strain used in this experiment was *Lactobacillus delbruechii* subsp. *bulgaricus* (LDSB) (preservation number AB200048); it was purchased from China Center for Type Culture Collection (Wuhan, Hubei, China).

Evaluation of the tolerance of LP-HFY15 to the gastrointestinal tract *in vitro*

First, 0.35 g protease (Nanjing Oddfoni Biological Technology Co., Ltd., Jiangsu, China) was added to 0.2 g NaCl (Chongqing Chuandong Chemical Co., Ltd., Chongqing, China) to prepare 100 mL simulated gastric juice. The pH of the solution was adjusted to 3.0, and then it was filtered and sterilized with a 0.45 µm filter. Then, 5 mL of the activated strain culture solution was taken and centrifuged at 3000 rpm for 10 min. The bacteria was collected and washed twice with sterile normal saline, and then 5 mL of normal saline was added to make a bacterial suspension. Then, 1 mL of the resuspension was inoculated in 9 mL of simulated gastric juice, shaken well, and placed in a 37°C incubator for 3 h, and the number of viable bacteria was measured at 0 h and 3 h. The survival rate was calculated using the following formula: Survival rate (%) = number of viable bacteria at 3 h (CFU/mL)/number of viable bacteria at 0 h (CFU/mL)×100 [2, 53].

Then, 2% of the inoculum amount was taken, and the overnight cultured bacteria solution was inoculated in an MRS-THIO medium (MRS medium containing 0.2% sodium thioacetate (Shanghai Macklin Biochemical Co., Ltd., Shanghai, China) containing 0.0% and 0.3% bovine bile salt. After incubating for 24 h at 37°C, with a blank medium (uninoculated MRS-THIO medium) as the control, the absorbance at 600 nm was measured with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA), and the tolerance to bile salts was calculated using the following formula: Growth efficiency (%)=(Bile salt medium OD₆₀₀)/(Blank medium OD₆₀₀)×100 [54].

Animal experiment design

Fifty 6-week-old male Kunming mice weighing 20 ± 5 g were purchased from the Experimental Animal Center of Chongqing Medical University (No. SYXK 2018-0003). All mice were fed standard feed and water under constant conditions in a light/dark cycle of 12 h at a temperature of 25 ± 2°C. After one week

of adaptive feeding, the mice were randomly divided into five groups: normal group, CCl₄-induced group (Chengdu Kelon Chemical Reagent Factory, Chengdu, Sichuan, China), silymarin group (Shanghai Yuanye Bio-Technology Co., Ltd. Shanghai, China), LP-HFY15 group, and LDSB group, with 10 mice in each group. The mice in the normal group and CCl₄-induced group were gavaged with 10 mL/kg saline per day; the mice in the silymarin group were given 50 mg/kg silymarin per day; the mice in the LP-HFY15 group were gavaged with 10⁹ CFU/kg LP-HFY15 per day. The mice in the LDSB group were given 10⁹/CFU kg LDSB every day, and the body weight of all the mice was measured and recorded for two weeks. Except for the normal group, the mice in all other groups were intraperitoneally injected with 0.8% CCl₄ (10 mL/kg) on the fourteenth day. After all the mice were fasted for 16 h, the mice were sacrificed (Fig. 8). The whole blood was centrifuged to separate the serum, and it was frozen and stored at -80°C. The liver was separated and weighed, and then freezed at -80°C or fixed with 4% formaldehyde solution.

Measurement of ALT, AST, TG, MDA, ROS, GSH, T-SOD, and CAT levels in mouse serum

The kits and instructions provided by Nanjing Jiancheng Institute of Biological Engineering (Nanjing, Jiangsu, China) were used to detect alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), malondialdehyde (MDA) levels, reactive oxygen species (ROS), glutathione (GSH), and total superoxide dismutation (T-SOD) and catalase (CAT) activity in serum enzyme.

Measurement of serum cytokines IL-6, TNF-α, and IFN-γ levels

Serum cytokines such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were detected in the serum using cytokine detection kits obtained from Shanghai Enzyme Link Biotechnology Co., Ltd, Shanghai, China.

Preparation of H&E stained sections of liver tissue

The mouse liver tissue was taken, soaked, and fixed with 10% neutral formalin, and then dehydrated in 95% ethanol for 24 h. Then, the tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histopathological analysis. Histopathological changes were observed under an optical microscope (BX43, Olympus, Tokyo, Japan), and the images were recorded.

Measurement of mRNA expression in mouse liver tissue (qPCR Measurement)

First, 50–100 mg of liver tissue was taken and placed in a homogenization tube equipped with small steel balls, and then 1 mL of Trizol reagent was added (Invitrogen, New York, USA) to separate and extract the total RNA from the liver homogenate. The concentration and purity of the total RNA were determined using a micro spectrophotometer (Nano-300, Hangzhou Allsheng Instruments Co., Ltd., Hangzhou, Zhejiang, China). Using the total RNA as a template, cDNA was synthesized by reverse transcription. First, 1 µL of cDNA was added to 2 µL of primers (Table 2), 10 µL of premix, and 7 µL of sterile ultrapure water into an eight-tube tube. Then, the mixture was denatured at 95°C for 3 min, annealed at 60°C for 20 s, and heated at 95°C for 1 min. The whole process was carried out for 40 cycles. Using GAPDH as the internal reference gene, the relative expression of mRNA of each target gene was calculated using the formula $2^{-\Delta\Delta C_t}$.

Table 2
Primers sequence list.

Gene	Forward Sequence	Reverse Sequence
SOD1	5'-AACCAGTTGTGTTGTCAGGAC-3'	5'-CCACCATGTTTCTTAGAGTGAGG-3'
SOD2	5'-CAGACCTGCCTTACGACTATGG-3'	5'-CTCGGTGGCGTTGAGATTGTT-3'
Nrf2	5'-CACATCCAGTCAGAAACCAGTGG-3'	5'-GGAATGTCTGCGCCAAAAGCTG-3'
NO-1	5'-TGCAGGTGATGCTGACAGAGG-3'	5'-GGGATGAGCTAGTGCTGATCTGG-3'
NQO1	5'-CCTGCCATTCTGAAAGGCTGGT-3'	5'-GTGGTGATGGAAAGCACTGCCT-3'
GSH	5'-AGCAGCTGCTGCACTCTACCAG-3'	5'-TGAACGCCGTCCGCATCCTCT-3'
CAT	5'-GGAGGCGGGAACCCAATAG-3'	5'-GTGTGCCATCTCGTCAGTGAA-3'
Bax	5'-GGGCCTTTTTGCTACAG-3'	5'-GACACTCGCTCAGCTTC-3'
Caspase-3	5'-CAGTGGATTCAAAATCC-3'	5'-ATATGCCCATTTTCAGGA-3'
Bcl-2	5'-GATGCTGGAGATGCGGA-3'	5'-AGACGTCCTGGCAGCCA-3'
IL-6	5'-ATGAAGTTCCTCTCTGCAA-3'	5'-AGTGGTATCCTCTGTGAAG-3'
TNF-α	5'-ATGGGGGGCTTCCAGAA-3'	5'-CCTTTGGGGACCGATCA-3'
IFN-γ	5'-GCTTTGCAGCTCTTCCTCAT-3'	5'-GTCACCATCCTTTTGCCAGT-3'
GAPDH	5'-TGACCTCAACTACATGGTCTACA-3"	5'-CTTCCCATTCTCGGCCTTG-3'

Statistical Analysis

Three measurements of serum and tissue samples were performed in parallel, and then the average value was calculated. SPSS software (SPSS v.25 for Windows, IBM Software Group, Chicago, IL, USA) was used to average and analyze the data. The Duncan multiple range test was used to evaluate the difference between the mean of each group by one-way analysis of variance. Differences with $p < 0.05$ were considered statistically significant.

Declarations

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Contributions

XL, FT and XZ designed research and supervised the study. XL and PW wrote the manuscript and interpreted data. YZ, QW and LR analyzed and interpreted data. All authors agree to be held accountable for the content therein and approve the final version of the manuscript.

Ethics declarations

Ethics approval and consent to participate

The animal study was reviewed and approved by the protocol for these experiments was approved by the Ethics Committee of Chongqing Collaborative Innovation Center for Functional Food (202101003B), Chongqing, China. The experimental process was in accordance with 2010/63/EU directive.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Figures

Figure 1

Observation of colony morphology and Gram stain of *L. plantarum* HFY15. (A) Colony morphology, (B) Gram staining result.

Figure 2

Tolerance of *L. plantarum* HFY15 to artificial gastric juice and bile salts.

Figure 3

ALT, AST activity, and TG content in mouse serum. a–e Mean values with different letters in the different bars are significantly different ($p < 0.05$) according to Duncan's multiple range test. CCl4-induced group: mice were injected with 10 mg/kg CCl4 on the 14th day; Silymarin group: mice were given 50 mg/kg silymarin every day and injected with 10 mg/kg CCl4 on the 14th day; LP-HFY15 group: mice were treated with 1.0×10^9 CFU/kg (b.w.) of *L. plantarum* HFY15 every day and injected with 10 mg/kg CCl4 on the 14th day; LDSB group: mice were treated with 1.0×10^9 CFU/kg (b.w.) of *L. delbruechii* subsp. *bulgaricus* every day and injected with 10 mg/kg CCl4 on the 14th day.

Figure 4

Mouse serum MDA level, SOD, CAT, GSH and ROS activity. a–e Mean values with different letters in the different bars are significantly different ($p < 0.05$) according to Duncan's multiple range test. CCl4-induced group: mice were injected with 10 mg/kg CCl4 on the 14th day; Silymarin group: mice were given 50 mg/kg silymarin every day and injected with 10 mg/kg CCl4 on the 14th day; LP-HFY15 group: mice were treated with 1.0×10^9 CFU/kg (b.w.) of *L. plantarum* HFY15 every day and injected with 10 mg/kg CCl4 on the 14th day; LDSB group: mice were treated with 1.0×10^9 CFU/kg (b.w.) of *L. delbruechii* subsp. *bulgaricus* every day and injected with 10 mg/kg CCl4 on the 14th day.

Figure 5

Expression levels of IL-6, TNF- α , and IFN- γ in mouse serum. a–e Mean values with different letters in the different bars are significantly different ($p < 0.05$) according to Duncan's multiple range test. CCl4-induced group: mice were injected with 10 mg/kg CCl4 on the 14th day; Silymarin group: mice were given 50 mg/kg silymarin every day and injected with 10 mg/kg CCl4 on the 14th day; LP-HFY15 group: mice were treated with 1.0×10^9 CFU/kg (b.w.) of *L. plantarum* HFY15 every day and injected with 10 mg/kg CCl4 on the 14th day; LDSB group: mice were treated with 1.0×10^9 CFU/kg (b.w.) of *L. delbruechii* subsp. *bulgaricus* every day and injected with 10 mg/kg CCl4 on the 14th day.

Figure 6

Observation of H&E staining of mouse liver.

Figure 7

mRNA expression of mouse liver-related genes. a–e Mean values with different letters in the different bars are significantly different ($p < 0.05$) according to Duncan's multiple range test. CCl4-induced group: mice were injected with 10 mg/kg CCl4 on the 14th day; Silymarin group: mice were given 50 mg/kg silymarin every day and injected with 10 mg/kg CCl4 on the 14th day; LP-HFY15 group: mice were treated with 1.0×10^9 CFU/kg (b.w.) of *L. plantarum* HFY15 every day and injected with 10 mg/kg CCl4 on the 14th day; LDSB group: mice were treated with 1.0×10^9 CFU/kg (b.w.) of *L. delbruechii* subsp. *bulgaricus* every day and injected with 10 mg/kg CCl4 on the 14th day.

Figure 8

Animal experiment design.