

Article

Protection Impacts of *Coix lachryma-jobi* L. Seed *Lactobacillus reuteri* Fermentation Broth on Hydrogen Peroxide-Induced Oxidative Stress in Human Skin Fibroblasts

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Abstract: This study researches the active ingredients in the fermented and aqueous extracts of three types of Coix seed. The results show that the active contents of total sugars, total phenols and total proteins of Coix seed fermented with *Lactobacillus reuteri*, *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus* are significantly higher compared to unfermented Coix seed aqueous extract (CW), and the free radical scavenging ability of Coix seed *Lactobacillus reuteri* fermented extract (CLRf) is significant. The protective effects of CLRf on hydrogen peroxide (H₂O₂)-induced oxidative stress in human skin fibroblasts (HSF) are investigated. The results show that cell viability, total antioxidant capacity, superoxide dismutase (SOD) activity, catalase (CAT) activity, collagen type I (COL-I) content and synthesis of hyaluronic acid (HA) significantly increased, and reactive oxygen species (ROS), matrix metalloproteinase-1 (MMP-1) content are decreased in CLRf-treated HSF compared to CW and damage model groups, providing effective protection to skin structure. The results show that CLRf can stimulate the PI3K/AKT signaling pathway, inhibiting intracellular ROS levels, positively regulating COL-I genes and significantly reducing MMP-1 expression, with anti-oxidative damage effects. As such, this study provides a theoretical basis for applying CLRf as a novel anti-oxidative agent in the cosmetics industry.

Keywords: Coix seed; *Lactobacillus reuteri*; fermentation broth; anti-aging; PI3K/AKT; human skin fibroblasts



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1. Introduction

Aging has always been a topic discussed by many scholars, and the free radical theory is one of the main theories to explain it [1]. Under normal circumstances, reactive oxygen species (ROS) in cells are always in the dynamic balance of production and elimination. It is involved in various life activities including gene transcription and protein synthesis. Long term exposure to the stimulus will rapidly increase the ROS content to the threshold that the antioxidant enzyme system cannot independently clear, and the oxidative stress reaction of the body begins to occur. Excessive free radicals begin to attack important biological macromolecules (nucleic acids, proteins, etc.) in cells, causing the loss of cell function and the decline of human body function, and eventually leading to the aging of the body [2,3]. Hydrogen peroxide (H₂O₂), the primary form of reactive oxygen species (ROS), is a by-product of cell metabolism which is involved in cell redox signaling. Excess H₂O₂ leads to lipid peroxidation and affects cellular senescence and apoptosis. H₂O₂ induces cellular damage and is one of the most widely used in vitro models of oxidative stress [4].

As the first defense barrier of human body, skin is always threatened by oxidative damage. Oxidative stress can lead to the increase of intracellular matrix metalloproteinases (MMPs) and the degradation of extracellular matrix (ECM) [5,6]. ECM is made up of a variety of matrix proteins that play an important role in cellular metabolism. The disruption

of ECM homeostasis and its protease dysfunction can lead to loss of skin intercellular lipids and disintegration of dermal support structures, and ECM homeostasis can be sustained by degradation and synthetic pathways. Decreased levels of proteoglycans in the ECM result in a reduction of the skin's ability to retain water and moisture, and a disruption of the normal structure and function of the skin. Collagen, a protein in the ECM, is essential for maintaining the physicochemical and mechanical properties of the skin. MMPs are a key group of proteases that disrupt the ECM [7–9]. Elevated levels of MMP expression trigger collagen degradation, disruption of antioxidant enzymes and expression of skin aging-related proteins, leading to skin aging, including loss of elasticity, wrinkles, dryness and hyperpigmentation [10].

Thus, it is thought that by promoting collagen synthesis in the skin and reducing MMP activity, the skin ageing process can be improved. Other studies have shown that phosphatidylinositol kinase (PI3K) plays an important regulatory role in collagen gene expression [11]. In addition, several downstream targets of PI3K, such as protein kinase B (AKT) are associated with collagen regulation, suggesting that the PI3K/AKT signaling pathway is closely related to skin aging due to oxidative stress [12].

Coix is a cereal plant of the family Gramineae which is widely grown in Asian countries. Coix seed is the mature kernel of *Coix lachryma-jobi* L., which is rich in various active nutrients, including proteins, amino acids, dietary fibre, fatty acids, polysaccharides and many vitamins, making it a dual-use crop for medicinal purposes [13]. It has potential for immunomodulation, reduction of cellular inflammation caused by lipoprotein oxidation, regulation of blood lipids, anti-obesity, antioxidation and allergic reaction inhibition [14–16], and has also been used in cardiovascular, diabetic, fatty liver and anti-cancer treatments [17–19].

Microbial fermentation is a green and environmentally friendly method of biotechnological transformation. Studies have reported that the fermentation broth of Coix seed with *Lactobacillus plantarum* has significantly higher nutritional activity components such as fatty acids, soluble dietary fibre and organic acids than unfermented Coix seed, which can promote the stability and prolong the storage time of Coix seed [20]. In addition, microbial fermentation techniques allow for the appropriate modification of cereal raw materials, which can enhance the bioavailability of active substances [21]. It has been shown that the fermentation of wheat and Coix seed by *Lactobacillus reuteri* and *Lactobacillus plantarum* can effectively enhance their antioxidant activity and inhibit the proliferation of cancer cells [22,23]. Although there has been considerable research into the benefits of fermented Coix seed, the effects of fermented Coix seed extracts on cellular oxidative stress are still yet to be explored. Therefore, the aim of this study was to investigate the modulating effect of fermented Coix seed on cellular oxidative stress and the prevention of skin aging due to oxidative damage.

This study selected *Lactobacillus bulgaricus*, *Saccharomyces cerevisiae* and *Lactobacillus reuteri* to ferment and prepare three kinds of Coix seed fermentation broth, using Coix seed aqueous extract (CW) as the control, and investigate the changes in the active substances and free radical scavenging ability of Coix seed after fermentation, and find the best strain. The effects of Coix seed fermentation broth on the activity of key enzymes for ROS scavenging and collagen synthesis in HSF, and the regulation of transcriptional levels of the PI3K/AKT signaling pathway, were investigated to research the protective effects of *Lactobacillus reuteri* fermented Coix seed on oxidatively damaged HSF.

2. Materials and Methods

2.1. Preparation of Coix Seed Fermentation Broth

Preparation of bacterial liquid: the YPD liquid medium was prepared by adding 1% yeast (Culture Collection Center of the Beijing Institute of Food and Brewing, Beijing, China) extract, 2% glucose and 2% peptone to 200 mL of water. After sterilisation at 121 °C for 30 min, a single colony of *Saccharomyces cerevisiae* was inserted into a shaker at 28 °C and rotated at 3000 g for 48 h to obtain *Saccharomyces cerevisiae*. The MRS broth

(Beijing Aoboxing Biotechnology Co., Ltd., Beijing, China) medium was prepared according to the manufacturer's instructions, sterilised at 121 °C for 30 min, and then inserted into a single colony of *Lactobacillus reuteri*, placed in a shaker at 37 °C and rotated at 3000 g for 48 h to obtain *Lactobacillus reuteri* solution. The *Lactobacillus bulgaricus* liquid is then obtained by dissolving the purchased *Lactobacillus bulgaricus* powder using sterile water.

Coix seed (Yunnan Baiyao Group Co., Ltd., Beijing, China) was pulverized with a high-speed multifunctional pulverizer and passed through a 50-mesh sieve to obtain a powder. To prepare Coix Seed *Lactobacillus reuteri* fermentation solution, 3 g of sieved Coix seed powder was weighed, deionised water was added at a ratio of 1:100 (g/mL), then sterilised at 121 °C for 30 min, added with 3% *Lactobacillus reuteri* solution after cooling, incubated for 15 h in a constant temperature incubator at 37 °C, sterilised at 110 °C for 30 min and centrifuged at $3000 \times g$ for 30 min, then the supernatant was removed from the membrane and set aside.

The Coix seed *Saccharomyces cerevisiae* fermentation broth (CSF) and *Lactobacillus bulgaricus* fermentation broth (CLF) was prepared in the same way as described above, by accessing 3% *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus*, respectively. Coix seed water extract (blank control) (CW): method as above without connection between strains. The absorbance of the three inoculated fermentation broths was measured at OD 600 nm, and subsequent experiments were performed when the absorbance reached 0.8–1.2.

2.2. Determination of Coix Seed Content

2.2.1. Total Sugar

According to the instructions of the total sugar kit (Solarbio Biotechnology Co., Ltd., Beijing, China), the concentration gradient of the standard was set and the standard curve was plotted with the concentration of the standard tube as the x-axis and the absorbance difference as the y-axis to obtain the standard equation $Y = 5.3871X + 0.1187$ ($R^2 = 0.9919$). Reagent I and reagent II were added to the sample solution to hydrolyze the total sugars to reducing sugars. An equal volume of Reagent III (a DNS reagent for the determination of reducing sugars) was added, mixed and allowed to stand and boiled in a water bath for 10 min. Then it was cooled to room temperature, triple distilled water was added and mixed well; 200 µL sample was taken and the absorbance was measured at 540 nm.

2.2.2. Total Phenol

The total polyphenol content of the three types of Coix seed fermentation broth and CW was determined by the forintanol method. The concentration gradient of Gallic acid was first set and the standard curve was plotted with the concentration and absorbance of Gallic acid as the horizontal and vertical coordinates, respectively, and the regression equation was obtained as $Y = 0.8591X + 0.0793$ ($R^2 = 0.9982$). 0.5 mL of the three Coix seed fermentations and WC was accurately weighed, the test was performed according to the same method, and absorbance at 765 nm was determined and substituted into the standard curve to calculate the total polyphenol content.

2.2.3. Total Protein

The instructions of the Bari-Polar BCA Protein Concentration Assay Kit (Biorigin Biotechnology Co., Ltd., Beijing, China) were followed. BCA and Cu^{2+} solution were used to prepare the BCA protein quantification solution at 50:1. Prepare a 96-well plate, add 200 µg of BCA Protein Quantitative Assay Solution and 20 µg of standard or sample, mix well and incubate for 1 h at 37 °C. The absorbance was measured at 562 nm and substituted into the standard curve to calculate the total protein content. The regression equation was $Y = 0.0015X + 0.0859$, $R^2 = 0.9951$, in which X was the total protein content (mg/mL) of the sample and Y was the absorbance of the sample.

2.3. DPPH Free Radical Scavenging Experiment

Dissolve 4 mg of accurately measured DPPH (Shanghai Maccabi Biochemical Technology Co., Ltd., Shanghai, China) in anhydrous ethanol and dilute to 50 mL to prepare 2×10^{-4} mol/L of DPPH solution for use. Samples: three different bacterial fermentations of Coix seed and its aqueous extract. 3 mL of different volume fractions were taken and mixed with an equal volume of DPPH solution, noted as tube A₁. 3 mL of DPPH solution was mixed with an equal volume of distilled water and recorded as tube A₂. 3 mL of different volume fractions of the sample was mixed with an equal volume of distilled water and recorded as tube A₃. After 30 min of reaction, the absorbance was measured at 517 nm using enzyme marker (Dickon Trading Co., Ltd., Shanghai, China), and the data obtained were used to calculate the scavenging capacity of three different bacterial fermentation broth and CW to DPPH free radicals. Inhibition rate = $[(A_2 + A_3 - A_1)/A_2] \times 100\%$.

2.4. Safety Determination of Coix Seed

2.4.1. Chicken Embryo Chorioallantoic Membrane Test (HET-CAM)

Fresh eggs (fertilized chicken embryo of white Leghorn chicken, Beijing Guichuan Yashen Breeding Center) weighing 50–60 g were chosen. According to the references [24], fertilised eggs were incubated for 9 days in an incubator at 38 °C and 60–70% humidity. The position of the air chambers was checked by illuminating the eggs, vascular-rich eggs were chosen and the shells were carefully peeled off with forceps to expose the surface of the CAM. Positive control group, negative control group and sample group (CW and CLRF) were set up respectively. Six eggs were selected for each group. 300 µL of 0.1 mol/L NaOH solution (positive control), 0.9% NaCl solution (negative control), CW and CLRF were added to CAM, respectively.

CAM vascular injury was observed and recorded after 3 min. This test used the endpoint evaluation method, and an endpoint score (ES) was calculated. Endpoint score (ES) includes three vascular response scores: bleeding, coagulation, and vascular melting. Bleeding refers to the flow of blood from the blood vessels or capillaries of the CAM. Coagulation is characterized by swelling of the vascular wall, clotting points inside and outside the blood vessels, and turbidity near the blood vessels. Vascular melting refers to vascular ablation on CAM membrane. According to the severity of the above three conditions, 0–3 points were awarded respectively. Six parallel sets of tests were conducted for each sample and the individual vascular response scores were calculated. The vascular response score with the highest total was used as the final ES score.

The ES scores were based on the phenomena of vascular haemorrhage and irritation classification, and the range of ES scores is shown in Table 1.

Table 1. ES Scoring Standards.

Rating Range	Irritation Classification
ES < 6	Non-irritating
6 < ES < 12	Mild irritation
12 < ES < 15	Moderate irritation
ES > 15	Severe irritation

2.4.2. Red Blood Cell Hemolysis Test

Fresh defibrinated rabbit blood (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) was taken and added with PBS buffer at a volume ratio of 10:4 (mL:mL). The supernatant was repeatedly washed and the precipitated cells were diluted with PBS buffer to a concentration of approximately 2% red blood cell suspension at an absorbance of $0.5 \pm 5\%$ at 560 nm to obtain a suitable concentration of red blood cell suspension (RBC). Different volume fractions of samples and RBC were mixed at a 3:1 ratio (750 µL sample + 250 µL RBC suspension) into 1.5 mL EP tubes, incubated for 60 min at 37 °C in a shaker and centrifuged for 1 min at $10,000 \times g$, and the supernatant was taken as A1. Negative control (A2): 750 µL PBS + 250 µL RBC suspension; complete hemolysis

control (A3): 750 μ L deionised water + 250 μ L RBC suspension. After centrifugation, the absorbance values of each group were measured at 560 nm and the data was recorded and saved. The hemolysis rates of the different samples were calculated as follows: hemolysis rate = $(A1 - A2)/(A3 - A2) \times 100\%$.

2.5. Cell Culture and Viability Experiment

Cell viability and protection against a model of H_2O_2 damage were explored by CCK8 assays with CLRF and CW. HSF cells from the same colony were used for this experiment. HSF (China Institute of Inspection and Quarantine, Beijing, China) were cultured in a flask containing DMEM medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA) and 1% penicillin-streptomycin (Thermo Fisher Scientific Co., Ltd., Shanghai, China). The cells were stored in a 37 °C HeraCell CO_2 incubator (Thermo Fisher Scientific Co., Ltd., Waltham, MA, USA) containing 5% CO_2 and cultured for 2 d.

100 μ L of HSF cells suspension (8×10^3 – 1×10^4 cells/mL) was added to each well of a 96-well plate (Corning, Corning, NY, USA) and pre-incubated for 12 h; then the medium was changed. Five concentrations of sample solutions of 0.25%, 0.5%, 1%, 2% and 5% were added to the sample wells, and serum-free DMEM was added to the control and blank wells, and incubated for 24 h. 10 μ L of CCK-8 (Biorigin Biotechnology Co., Ltd., Beijing, China) was then added to each well and they were incubated for a further 3 h. Cell viability was calculated by measuring absorbance at 450 nm, and cell activity was observed after CLRF and CW treatment.

After 24 h of incubation, a model of H_2O_2 cell damage was established, and three concentration gradients of 0.5%, 1%, and 2% volume fractions of H_2O_2 were chosen. After 2 h of cell damage, wash twice with PBS and add serum-free DMEM. After that, 10 L of CCK-8 was added to each well and incubated for another 3 h. Cell viability is determined at 450 nm, and the protective effects of CLRF and CW on HSF are observed. Cell viability was calculated according to the following formula:

Cell viability (%) = (measurement well absorbance values-blank control absorbance values)/(cell control absorbance values-blank control absorbance values) * 100%.

At the appropriate damage concentration, HSF was inoculated in 6-well plates at a density of 5×10^5 cells/well. After 24 h of culture in a 37 °C and 5% CO_2 incubator, the medium was discarded and washed twice with PBS. In the sample wells, add 2 mL of CLRF and CW (blank controls were treated with serum-free DMEM) and incubate for 24 h. The medium was discarded and PBS was cleaned twice after being damaged by the addition of 1000 μ g/mL hydrogen peroxide for 2 h. After 12 h of culture in an incubator set at 37 °C with 5% CO_2 , add serum-free DMEM. Then, remove the media and wash it twice with PBS. Add 200 μ L of lysis buffer and use a cell scraper to lyse the cells for subsequent assays.

2.6. Measurement of Total Antioxidant Capacity

The collected cells were used and centrifuged at $12,000 \times g$ for 5 min at 4 °C, and the supernatant was extracted to determine the total antioxidant capacity of CLRF and CW after H_2O_2 -induced damage, according to the ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) Assay Kit (Beyotime Biotechnology Co., Ltd., Shanghai, China). Trolox was chosen as the standard solution, a vitamin E analogue with similar antioxidant capacity to vitamin E. The total antioxidant capacity of HSF cells after H_2O_2 -induced damage was then determined according to the ABTS Assay Kit. The absorbance was measured at 734 nm using an enzyme marker (Dickon Trading Co., Ltd., Shanghai, China), and the total antioxidant capacity was calculated according to the standard curve.

2.7. Measurement of Intracellular ROS Levels

After HSF cells were damaged by H_2O_2 , they were incubated with CLRF at 2.00% *v/v* for 24 h. The sample solution was decanted and washed twice with PBS. The fluorescent probe DCFH-DA was diluted with serum-free medium 1:1000, and 2 mL was added to each

well. The subsequent steps were carried out according to the procedure of the ROS Assay Kit and the fluorescence intensity of intracellular ROS in CLRF and CW after treatment was measured at 488 nm excitation wavelength and 525 nm emission wavelength using an enzyme marker (Dickon Trading Co., Ltd., Shanghai, China). Cells were collected and the total protein content of the different wells was determined based on the BCA kit (Biorigin Biotechnology Co., Ltd., Beijing, China). Used as a standard for determining ROS fluorescence intensity.

2.8. ELISA

HSF was inoculated in 6-well plates at a density of 5×10^5 cells/well. After 24 h of culture in a 37 °C and 5% CO₂ incubator, the medium is poured out and washed twice with PBS. In the sample wells, add 2 mL of CLRF and CW (controls were treated with serum-free DMEM) and incubate for 24 h. The medium was discarded and the model and sample groups were disrupted by addition of 1000 µg/mL hydrogen peroxide for 2 h and then washed twice with PBS and serum-free DMEM was added. After 12 h of culture in an incubator set at 37 °C with 5% CO₂, the cell supernatant collected. Add 200 µL of lysis buffer and use a cell scraper to lyse the cells and collect the cell lysate.

Collected cell supernatant or lysate was combined with antibodies or antigens fixed in the kit. The non-conjugates were removed by washing the plate, and enzyme-labeled antigens or antibodies were added. The amount of enzyme that can be fixed is related to the amount of substance detected in the sample for quantitative analysis. The detailed operation was analyzed according to SOD, CAT, MMP-1, HA and COL-I ELISA kits (Wuhan Huamei Biological Engineering Co., Ltd., Wuhan, China; Nanjing Jiancheng Institute of Bioengineering, Nanjing, China).

2.9. qRT-PCR

HSF was inoculated into 6-well plates at a density of 5×10^5 cells/well and serum DMEM was incubated for 24 h. The sample group was replaced with 2 mL CLRF and CW, respectively, and the control and model groups were treated with serum-free DMEM and incubated for 24 h. The sample and model groups were damaged by addition of 1000 µg/mL hydrogen peroxide for 2 h, then washed twice with PBS and incubated with serum-free DMEM for 12 h. Cells from the control, model and sample groups were collected, and RNA was extracted from HSF using the instructions for Trizol (total RNA extraction reagent). Extracted RNA was stored in a refrigerator at −80 °C. The regulation of intracellular signalling pathways by CLRF and CW was explored by qRT-PCR. The cDNA first-strand synthesis reaction was performed using a TINAGEN FastQuantRT Kit and FastQuant cDNA First-strand Synthesis Kit. Based on gene sequences obtained from the National Center for Biotechnology Information (NCBI), β-actin was used as the reference gene, specific primers were shown in Table 2. The reaction conditions were set up for operation according to the instructions of the Transgenic® Top Green qPCR Super Mix Kit, and the qRT-PCR instrument (QuantStudio 3, ThermoFisher, Shanghai, China) was analysed experimentally. The control group transcript level was set to 1. The mRNA transcript levels of the model and sample groups were compared to the control group, and the difference in mRNA expression between the cells in each group and the control group was calculated.

Table 2. Primers.

Primers	Direction	Primer Sequence (5'-3')
β-actin	F	TGGCACCAGCACAATGAA
	R	CTAAGTCATAGTCCGCCTAGAAGCA
SOD	F	TGGAGATAATACAGCAGGCT
	R	AGTCACATTGCCCAAGTCTC
CAT	F	CCTTCGACCCAAGCAA
	R	CGATGGCGGTGAGTGT

Table 2. Cont.

Primers	Direction	Primer Sequence (5'-3')
MMP-1	F	TTGAGAAGCCTTCCAACCTCTG
	R	CCGCAACACGATGTAAGTTGTA
COL-I	F	CCTGGTCCTCCTGGTAGT
	R	TCCCTTCTCTCCTGGTTG
HAS 1	F	TACTTTGGGGATGACCGGC
	R	AAGTACGACTTGGACCAGCG
PI3K	F	TCATAGCCACTGATCCACTT
	R	AAGGGTGACTTTTGGTAAACAT
AKT	F	ACTGTCATCGAACGCACCTT
	R	CTCCTCCTCCTCCTGCTTCT

2.10. Statistical Analysis

Three separate experiments were conducted, each of which was the subject of three technical reports and analyses. The experimental data was counted using Excel software (Version 2211 Build 16.0.15831.20098), with the experimental results expressed as mean $\pm$ standard deviation (SD). GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA) was used for data visualization. *T*-test was used to determine the significance of differences between the groups (^{ns} $p > 0.05$, #, * $p < 0.05$; ##, ** $p < 0.01$, ###, *** $p < 0.001$). Adobe illustrator was used to draw the mechanism.

3. Results

3.1. Determination of Fermentation Broth Content of Three Species of Coix Seed

The microbial fermentation method was used to promote the release of active substances from Coix seed by three different bacteria to ferment Coix seed. As shown in Table 3 and Figure 1A–C, the study analyzed the content of total sugar, total phenol, and total protein in CLF, CSF, CLRF and CW.

Table 3. Active substance content of the fermentation broth of three species of Coix Seed.

Sample	Total Sugar (mg/mL)	Total Phenol (mg/mL)	Total Protein (mg/mL)
CW	1.39 $\pm$ 0.05	3.48 $\pm$ 0.03	2.34 $\pm$ 0.01
CLF	3.63 $\pm$ 0.03	3.58 $\pm$ 0.11	2.05 $\pm$ 0.01
CSF	1.96 $\pm$ 0.06	4.45 $\pm$ 0.02	2.56 $\pm$ 0.01
CLRF	4.69 $\pm$ 0.06	4.69 $\pm$ 0.10	2.44 $\pm$ 0.01

Figure 1A shows the content of total sugar in the three Coix seed fermentation broths and CW, and the content of total sugar in CLF and CLRF was 2.61 and 3.37 times higher than CW, respectively. Figure 1B,C show the contents of total phenol and total protein in the three Coix seed fermentation broths and CW. Compared with CW, the total phenol and total protein contents of CSF and CLRF were significantly increased, with CLRF showing superior results. Compared to CLF and CSF, CLRF was more effective in enhancing the main active substances in Coix seed ($p > 0.001$). The experimental results showed that the fermentation of *Lactobacillus reuteri* could significantly increase the content of the main active substances, total sugar, total phenol and total protein in the product, thereby enhancing its efficacy.

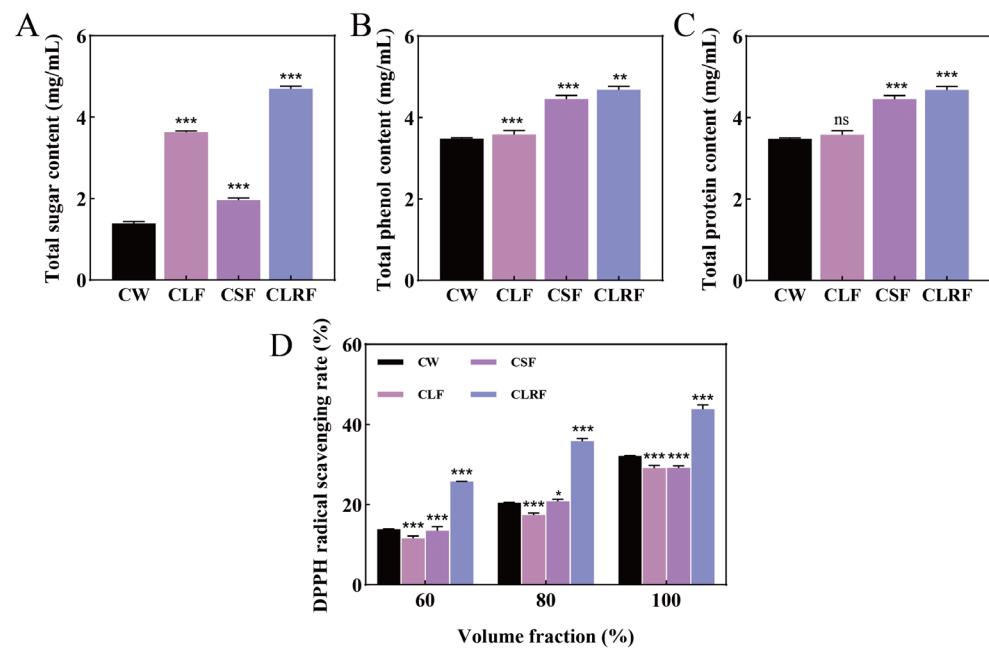


Figure 1. Determining the active substance content and DPPH radical scavenging capacity of three different Coix seed fermentation broths: (A) Total sugar, (B) Total phenol, (C) Total protein and (D) DPPH radical scavenging capacity of three different volume fractions of Coix seed fermentation broth. CW: Coix seed water extract; CLF: Coix seed *Lactobacillus bulgaricus* fermentation broth; CSF: Coix seed *Saccharomyces cerevisiae* fermentation broth; CLRf: Coix seed *Lactobacillus reuteri* fermentation broth. Each value is expressed as mean $\pm$ SD ($n = 3$). The difference analysis between other experimental groups and the CW group is indicated by “*” (ns > 0.05, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, compared with CW group).

3.2. DPPH Free Radical Scavenging Experiment of Three Coix Seed Fermentation Broths

DPPH radical scavenging assay is a common in vitro test for antioxidant properties. The scavenging effects of DPPH radicals are shown in Figure 1D. It can be seen that the scavenging effects of CLRf on DPPH radicals were significantly higher than those of CLF, CSF and CW, the scavenging rate tended to increase with the increased volume fraction of the sample, and the scavenging rate of DPPH radicals by CLRf was nearly 50% when the volume fraction was 100%. The ability of CLF and CSF to scavenge DPPH radicals is essentially lower than CW.

3.3. Safety Determination of CLRf

3.3.1. Chicken Embryo Chorioallantoic Membrane Test

Vascular-rich eggs were selected for the experiment, and the shells were carefully peeled from the air chambers with forceps to expose the surface of the CAM, washed with NaCl solution, dropped into 300 μ L of positive control, negative control and sample, and observed for 3 min. Morphological observations and scores were made for the positive control (0.1 mol/L sodium hydroxide solution), negative control (0.9% NaCl solution), CW and CLRf before and after the addition of CAM samples. The results of the scoring are shown in Table 4, with no irritation at either CLRf or CW. It is clear from Figure 2A that the CAM vascular response of the positive control was accompanied by significant haemorrhage and vascular lysis, while the CAM vascular responses of CW and CLRf were consistent with the negative control in that there was no haemorrhage, vascular coagulation or vascular lysis, and therefore neither CW nor CLRf caused irritation.

Table 4. Results of Eye Irritation of Chicken Chorioallantoic Membrane.

Samples	Haemolysis	Numbering						ES
		1	2	3	4	5	6	
Positive control (0.1 mol/L NaOH solution)	Bleeding	2	2	2	2	2	2	12
	Vascular coagulation	2	1	1	2	1	2	
	Vasolysis	1	1	1	1	1	1	
Negative control (0.9% sodium chloride solution)	Bleeding	0	0	0	0	0	0	0
	Vascular coagulation	0	0	0	0	0	0	
	Vasolysis	0	0	0	0	0	0	
CW	Bleeding	0	0	0	0	0	0	0
	Vascular coagulation	0	0	0	0	0	0	
	Vasolysis	0	0	0	0	0	0	
CLRf	Bleeding	0	0	0	0	0	0	0
	Vascular coagulation	0	0	0	0	0	0	
	Vasolysis	0	0	0	0	0	0	

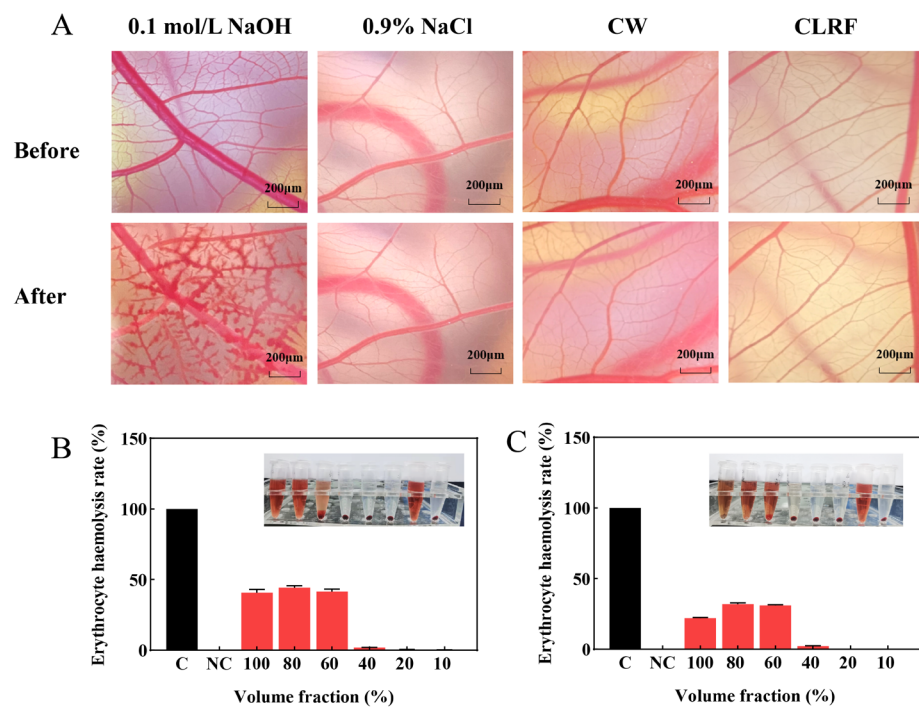


Figure 2. Safety measurements of CW and CLRf: (A) Vascular presentation and response plots before and after representative CAM spiking for CW and CLRf; (B) Erythrocyte hemolysis rates for different volume fractions of CW; (C) Erythrocyte hemolysis rates for different volume fractions of CLRf. In the photographs in Figure 2B and C, the red colour of the solution in the centrifuge tube correlates positively with erythrocyte haemolysis. The supernatant of the centrifuge tube is colourless and clear with a red precipitate at the bottom, indicating low or no haemolysis of the sample. CW: Coix seed water extract; CLRf: Coix seed *Lactobacillus reuteri* fermentation broth. Each value is expressed as mean $\pm$ SD ($n = 3$).

3.3.2. Red Blood Cell Hemolysis Test

In addition to the chicken embryo chorioallantoic membrane test, the erythrocyte hemolysis test is also a classic safety test to check sample irritation. Figure 2B,C show a bar graph of the hemolysis rates of CW and CLRf at different concentrations. The centrifuge tubes from left to right are, in order of volume fraction, 100%, 80%, 60%, 40%, 20% and 10% of the sample solution, positive control for complete hemolysis and negative control. When the volume fraction was greater than 60%, the erythrocyte hemolysis rate was around 40% for CW and 30% for CLRf, and when the volume fraction was less than 40%, the

erythrocyte hemolysis rate was significantly reduced to less than 3% for both CW and CLRF. Experiments have shown that CW is more irritating than CLRF at high volume fractions, and that both CW and CLRF are less irritating at low volume fractions.

3.4. Effects of CW and CLRF on HSF Activity

CCK-8 assay was widely used for cell proliferation and cytotoxicity assays, and was able to examine the effect of the sample on cell viability. Figure 3A shows the effect of different volume fractions of CLRF and CW on HSF activity. When the sample volume fraction was between 0.25% and 2%, the HSF survival rate was above 85% and the cell survival rate increased with the sample volume fraction. At sample volume fractions of 1% and 2%, CLRF and CW had proliferative effects on HSF. CLRF was less cytotoxic to HSF than CW, with a highly significant difference between CLRF and CW at a volume fraction of 2% ($p < 0.001$).

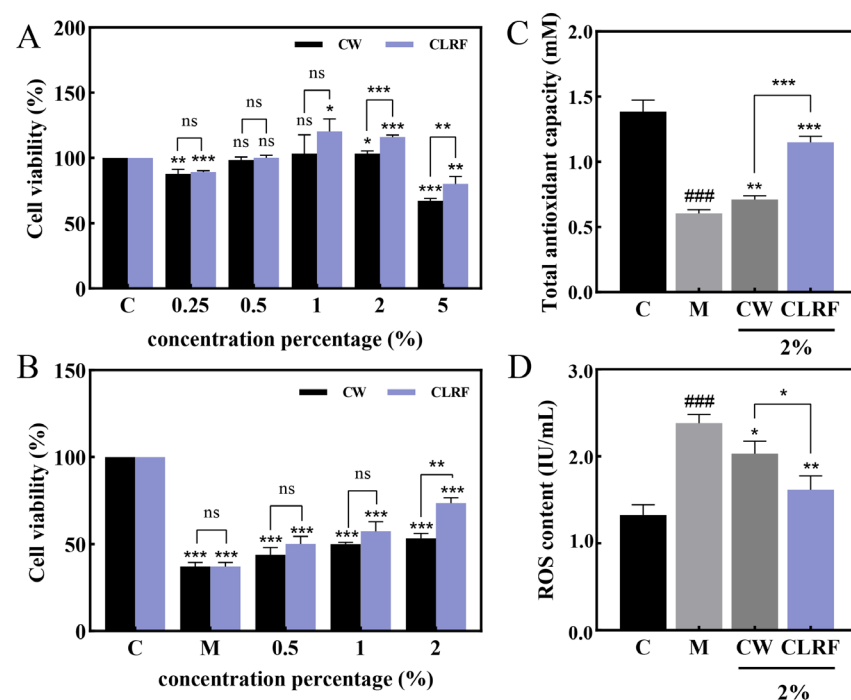


Figure 3. Effects of CLRF and CW on HSF cells viability total antioxidant capacity and ROS content: (A) Effects of different concentrations of CLRF and CW on cell viability; (B) Protective effects of different concentrations of CLRF and CW on HSF cells after H₂O₂-induced damage; (C) Total antioxidant capacity of CLRF and CW; (D) Ability of CLRF and CW to scavenge ROS. CW: Coix seed water extract; CLRF: Coix seed *Lactobacillus reuteri* fermentation broth; C: control group; M: H₂O₂ damage model group. Each value is expressed as mean $\pm$ SD ($n = 3$). (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns > 0.05 , versus model group, ###: $p < 0.001$, versus control group).

The protective effects of CLRF and CW on H₂O₂-induced damage to HSF at 0.5%, 1% and 2% volume fractions were examined, as shown in Figure 3B. Cell survival was dramatically reduced after H₂O₂ damage. The protective effects of CLRF and CW were found to increase with the increase in volume fraction compared to the damage model group. Overall, the protective effects of CLRF on the cells were stronger and there was a highly significant difference between CLRF and CW at a volume fraction of 2% ($p < 0.001$). In conclusion, considering the growth conditions of the cells, CLRF and CW at a volume fraction of 2% were used for subsequent experiments.

3.5. Total Antioxidant Capacity and Active Oxygen Detection

The total antioxidant capacity of CLRF and CW on H₂O₂-induced damaged HSF at 2% *v/v* was investigated, and the results are shown in Figure 3C. Compared to the control group, the total antioxidant capacity of H₂O₂-induced damaged HSF was significantly reduced. However, cells treated with CLRF and CW were protected compared to the damage model group. CW achieved a scavenging rate of 0.71 mM Trolox equivalent for ABTS+. CLRF achieved a scavenging rate of 1.15 mM Trolox equivalent for ABTS+, which was 1.61 times and 1.90 times that of CW and model group, respectively. The protective effects of CLRF on damaged cells were highly significant ($p < 0.001$).

H₂O₂ damage induces excessive ROS in cells, destroys the balance between oxidation and antioxidation in vivo, and induces oxidative stress, leading to cell aging [25]. The effects of CLRF and CW on HSF ROS content after H₂O₂-induced injury at 2% volume fraction were investigated, and the results are shown in Figure 3D. Compared to the control group, the cells showed a 1.79-fold increase in intracellular ROS content after H₂O₂ damage. However, both CLRF and CW treated cells showed a decrease in ROS compared to the model group, and CLRF was the most effective in reducing the ROS content of HSF cells.

3.6. Effects of CLRF and CW on Antioxidant and Protein Hydrolase Enzymes and Their Gene Expression

This article further tested the effects of CLRF and CW on the enzymatic activities of intracellular antioxidant and protein hydrolases, collagen, matrix metalloproteinases and hyaluronic acid content, and analysed their mRNA expression. As shown in Figure 4, the model group that underwent H₂O₂-induced damage showed that the enzymatic activity and mRNA expression of the antioxidant enzymes SOD and CAT were reduced by nearly half compared to the control group. Compared to the model group, both CLRF and CW significantly increased the enzymatic activities and mRNA expression of SOD and CAT at 2% volume fraction, and the effects of CLRF were more significant. Cells treated with CLRF and CW showed a significant increase in the activity of protein hydrolases COL-I and HA, and a more than threefold increase in mRNA expression. In addition, the enzyme activity of MMP-1 was significantly reduced, and the mRNA expression level of CLRF-treated cells was close to that of the uninjured cell model.

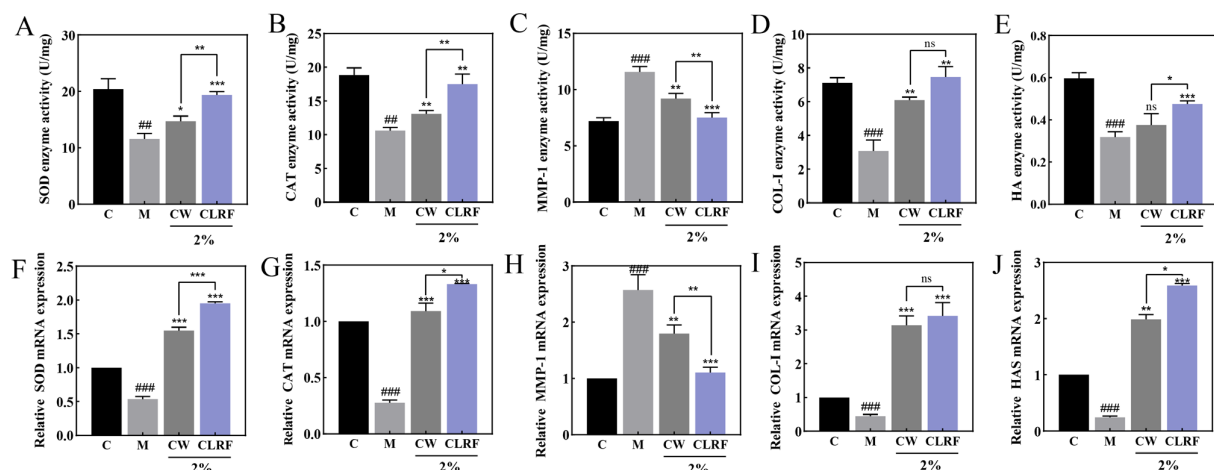


Figure 4. Effects of CLRF and CW on HSF antioxidant enzyme and protein hydrolase content: Effects of CLRF and CW on SOD content (A) and its gene expression (F); Effects of CLRF and CW on CAT content (B) and its gene expression (G); Effects of CLRF and CW on MMP-1 activity (C) and its gene expression (H); Effects of CLRF and CW on COL-I content (D) and its gene expression (I); Effects of CLRF and CW on HA content (E) and HAS 1 gene expression (J). CW: Coix seed water extract; CLRF: Coix seed *Lactobacillus reuteri* fermentation broth; C: control group; M: H₂O₂ damage model group. Each value is expressed as mean $\pm$ SD ($n = 3$). (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns > 0.05 , versus model group, ##: $p < 0.01$, ###: $p < 0.001$, versus control group).

3.7. Regulation of PI3K/AKT Signaling Pathway

PI3K/AKT is involved in regulating cell proliferation, apoptosis and other physiological activities, and is a critical pathway associated with oxidative stress [26]. As shown in Figure 5, the expression of the PI3K/AKT gene was significantly decreased in HSF after H₂O₂ treatment compared to the control group. Compared to the model group, the CLRf and CW were able to upregulate the expression of PI3K/AKT signaling pathway. In addition, the up-regulation of PI3K/AKT gene expression by CLRf was significantly higher than that by CW.

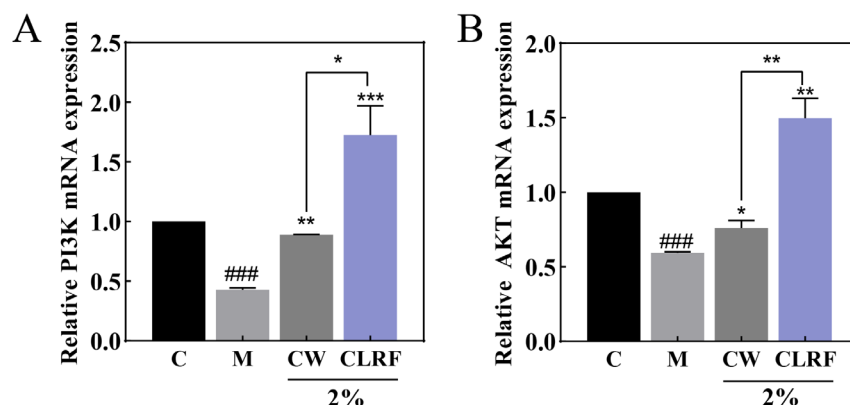


Figure 5. Expression of PI3K/AKT pathway genes in cells at 2% concentration of CLRf and CW: (A) Relative expression of PI3K in HSF cells; (B) Relative expression of AKT mRNA in HSF cells. CW: Coix seed water extract; CLRf: Coix seed *Lactobacillus reuteri* fermentation broth; C: control group; M: H₂O₂ damage model group. Each value is expressed as mean $\pm$ SD ($n = 3$). (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ versus model group, ###: $p < 0.001$, versus control group).

4. Discussion

In this study, the three different strains *Lactobacillus bulgaricus*, *Saccharomyces cerevisiae* and *Lactobacillus reuteri* were used to ferment Coix seed, and the differences in active substance content and in vitro free radical scavenging ability in the fermentation broths were investigated. *Lactobacillus reuteri* was finally selected as the best fermenting strain, and its activity in CLRf and CW in reducing skin aging caused by oxidative stress and differences were investigated at the biochemical, cellular and molecular levels.

Studies have proven that Coix seed are rich in a variety of active ingredients that can prevent cellular oxidative stress and have anti-oxidant effects [13,27]. Fermentation of Coix seed can increase the content of active ingredients and enhance antioxidant and other activities [23]. The results of the biochemical investigation show that all three Coix seed fermentation solutions can effectively increase the active substance content of Coix seed, including total sugar, total phenol, and total protein, probably because the fermentation treatment of Coix seed is accompanied by structural or molecular weight changes that increase the active substance content. However, the specific changes of active substances in the fermentation process need further study. The three fermentation solutions also showed different degrees of antioxidant activity, with CLRf having a higher content of active substances and a superior ability to scavenge free radicals. In this study, we established a model of H₂O₂-induced HSF damage to explore the protective effects and mechanism of CLRf and CW against oxidative stress-induced skin aging damage.

Under normal physiological conditions, ROS maintain a dynamic balance between the oxidation and antioxidation systems, and are important regulators of intercellular signalling. The increase in ROS levels leads to oxidative stress in skin cells, which is believed to be a precursor to premature skin aging [13,23]. Excessive accumulation of ROS disturbs the balance between oxidative and antioxidant systems and enhances the synthesis of MMP-1, which breaks down collagen type I (COL-I) and causes the accompanying degradation of HA in ECM [28,29]. Studies have shown that Coix lacryma has a protective effect

against oxidative stress, reducing oxidative stress markers in rat serum and improving its antioxidant capacity [30].

It was found that after H₂O₂-induced damage, the content of ROS and the enzymatic activities of SOD and CAT were significantly increased in HSF cells. However, HSF pretreated with CW and CLRf, with reduced ROS content, had a protective effect against cellular oxidative stress. The activities of intracellular antioxidant enzymes and protein hydrolases were increased, with the effect of CLRf being more significant. In addition, compared to CW, CLRf were able to inhibit the synthesis of MMP-1, protect COL-I and HA from catabolism more effectively, maintain the stability of extracellular matrix structure, and reduce the decomposition and adhesion of collagen, alleviate the disintegration of dermal support structure, and effectively slow down the dermal structure damage and skin aging caused by H₂O₂ stimulation.

To further investigate the protective mechanisms of CLRf and CW against antioxidant in HSF, we examined the effects of CLRf and CW on the transcriptional levels of key genes in the PI3K/Akt signaling pathway.

Bujor investigated the role of AKT in the production of COL-I in HSF and its role in systemic sclerosis fibrosis, and found that AKT is a positive regulator of collagen gene expression and a negative regulator of synthetic MMP-1 in HSF [12]. Blocking AKT inhibited the production of COL-I and led to the upregulation of MMP-1. AKT is a core element of the PI3K/AKT signalling network. This study also confirmed that CLRf and CW can effectively inhibit the upregulation of MMP-1 expression triggered by oxidative stress through activating the PI3K/AKT signalling pathway, contributing to the synthesis of cellular COL-I and the significant upregulation of hyaluronan synthetase 1 (HAS 1) expression.

It has also been shown that PI3K/AKT often works with transcription factor NF-E2-related factor 2 (Nrf2) to regulate cellular damage caused by oxidative stress, and the activation of this pathway can slow down the aging of the body. The antioxidant genes SOD, CAT and GSH-Px are downstream genes of the Nrf2-Keap-1 pathway, whose main role is to scavenge ROS and protect cells from oxidative damage [31]. Zhang [32] found that purple rivet flavonoids could activate the PI3K/AKT signaling pathway, affecting Nrf2-mediated Mn SOD gene expression and upregulated SOD protein expression, and thereby reducing mitochondrial oxidative stress [33]. This study shows that the expression of ROS protein was significantly reduced in HSF cells treated with the sample group, and the expression of SOD and CAT antioxidant enzyme mRNA was significantly enhanced. This suggests that CLRf and CW may regulate cell proliferation by activating the PI3K/AKT pathway, and inhibit intracellular ROS expression due to oxidative stress. However, whether Nrf2 mediates this and whether CLRf and CW can have protective effects against mitochondrial oxidative stress needs further validation.

In summary, CLRf and CW have strong protective effects against H₂O₂-induced HSF damage and great application potential in improving skin aging damage due to oxidative stress. As shown in Figure 6, our study demonstrated that CLRf significantly reduced intracellular ROS levels, increased total cellular antioxidant capacity and slowed MMP-1-mediated COL-I degradation, contributing to HA synthesis and mitigating skin aging. Furthermore, in the exploration of the antioxidant mechanism, it was found that both CLRf can enhance the cellular antioxidant system and relieve skin aging by activating the PI3K/AKT signaling pathway and increasing the expression of antioxidant enzymes and hydrolytic proproteins.

H₂O₂-induced oxidative HSF cellular damage and macroscopic skin aging are multifaceted, and there are some limitations in this study. This experiment did not verify the main indicators of cellular loss of function and senescence at the cellular level. Another study showed that H₂O₂-induced oxidative stress causes DNA damage in HSF cells, causing telomere shortening and reduced telomerase activity in HSF cells [34]. This is an issue worthy of in-depth consideration in subsequent experimental investigations, which can verify the preventive effect of Coix Seeds on HSF cells aging in many ways.

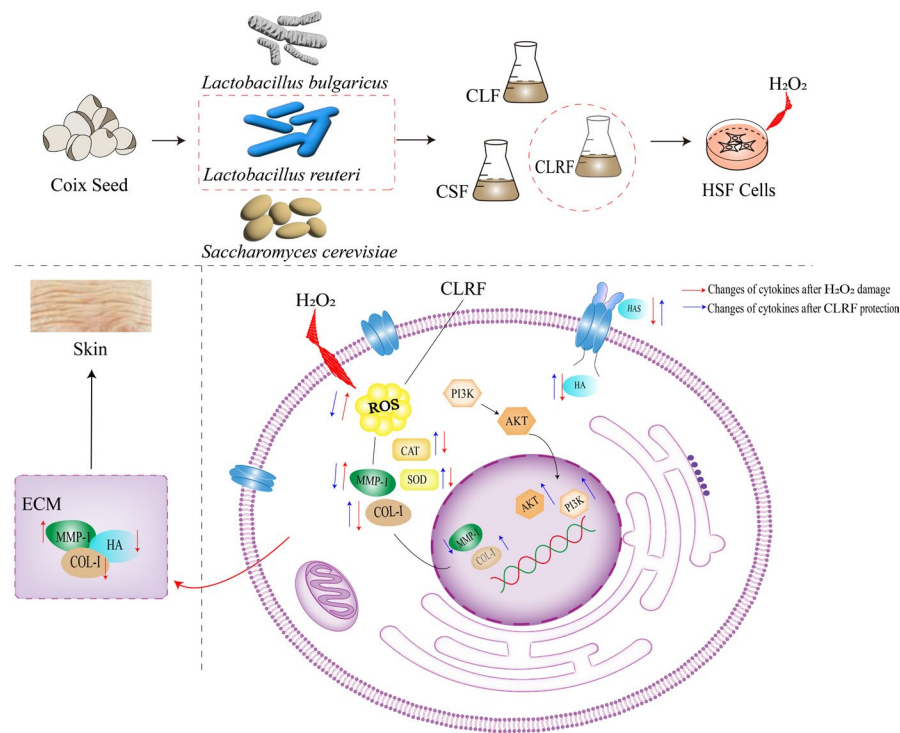


Figure 6. A schematic diagram showing the regulatory mechanisms of the protective effects of CLRF on skin fibroblasts damaged by H₂O₂. Coix seeds were fermented by three bacteria and finally CLRF was selected for cellular and molecular level investigation. After the protective treatment with CLRF, the HSF cells activated the expression of PI3K/AKT signaling pathway, inhibited the accumulation of ROS in the cells, enhanced the cellular antioxidant system, increased the content of COL-I and HA in the cytoplasmic matrix and decreased the content of MMP-1, protected the skin structure and slowed down the damage of H₂O₂ on the HSF cells.

5. Conclusions

Through biochemical, cellular and cellular molecular analyses, it was shown that Coix seed can promote antioxidants in cutaneous fibroblasts, protect the skin mitigate oxidative stress-induced damage to skin support structures and the aging process and activate the transcriptional level of the PI3K/AKT signaling pathway by regulating antioxidant enzymes and proteases. It can also inhibit the expression level of MMP-1 mRNA and up-regulate the expression of COL-I, providing a theoretical basis for applying Coix seed as an anti-aging ingredient in cosmetics. However, additional studies, such as histology, animal experiments and other techniques, are needed to elucidate the underlying mechanisms.

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