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Study on the efficacy of Himalayan native probiotics JMB326 in skincare and photoprotection

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Abstract

The skincare effect of probiotics is in the hotspot of active ingredient development. *Lactobacillus reuteri* is a safe and reliable functional probiotic, which has been widely used in medicine, and health food and has great potential in beauty and skincare. In this study, we found that *L. reuteri* fermentation filtrate(LrFF) of Himalaya probiotic strain JMB326 significantly reduced the accumulation of intracellular reactive oxygen species (ROS) by 78.4% (***) at 5% (v/v) and 77.9% (***)) at 10% (v/v), increased mitochondrial membrane potential (JC-1), and reduced cellular DNA damage induced by UV exposure. In summary, LrFF-JMB326 can recover the skin cells from Ultraviolet. The results of this study can be used as theoretical support for the future application of this material in food and cosmetics.

1. Introduction

As the largest organ of the human body, the skin's main function is to provide barrier protection for the human body. In daily life, skin exposure to ultraviolet radiation is inevitable. Although proper exposure to sunlight is beneficial to human health, the damage of ultraviolet rays to the skin cannot be ignored. Therefore, in recent decades, the global focus on UV protection and skin photo-aging has continued to attract attention.

Oxidative stress occurs when cells are exposed to ultraviolet radiation [1], the main sources of ROS include the electron transport chain of mitochondria, ultraviolet radiation and blue light. ROS can damage key proteins such as collagen and elastin, leading to sagging skin and more wrinkles [2]. ROS attacks DNA in skin cells, causing mutations and abnormal gene expression that accelerate aging [3]. The mitochondrial membrane potential is the electrochemical potential difference between the two sides of the inner mitochondrial membrane due to the proton gradient, which usually appears as a negative voltage difference between the inside and the outside, ranging from about -180 to -220 mV [4]. For example, Yousuke Toduka *et al.* [5] proposed that metal oxide nanoparticles, such as CuO, ZnO, and NiO, cause DNA damage and form γ-H2AX focal points by increasing intracellular ROS levels. It is an important indicator of mitochondrial function, which directly reflects the oxidative

phosphorylation capacity of mitochondria, the efficiency of ATP production and the energy state of cells. Multiple studies have clearly shown that excessive production of ROS causes DNA damage, which triggers the formation of γ-H2AX.

L. reuteri is a beneficial group of bacteria that colonizes the intestinal tract of mammals and has been reported to have excellent skin protective effects in many literatures. *L. reuteri* can enhance skin barrier function and wound healing ability, and also regulate immune cells to secrete cytokines to relieve skin inflammation [6]. Zhao J et al. found that one type of *L. reuteri* byproducts has strong antioxidant properties to skin cells, as well as the ability to clear free radicals and lipid oxides.

These studies directly prove that *L. reuteri* has a very promising future in the application of skin cosmetic raw materials with UV antioxidants as the core. The strain of *L. reuteri* JMB326 used in this study was collected, isolated and purified in the Himalayan region of China. Strains domesticated in extreme environments usually have strong stress resistance, which indirectly predicts that they have unmatched antioxidant and UV resistance.

2. Materials and Methods

2.1 Experimental Materials

NHEK (Normal Human Epidermal Keratinocytes, Female, aged 50, Face), NHDF (Normal Human Dermal Fibroblast, aged 48, Face), HaCAT (Human Immortalized Epidermal Cells), FBS (Fetal Bovine Serum, REF: 04-00101ACS, America), BCS (Bovine Calf Serum supplement, Hyclone, SH30027.03, AAL211105, America). DMEM (Dulbecco's Modified Eagle Medium, L-glutamine, 110 mg/L sodium pyruvate and phenol red, HEPES-free, China), 0.05% Trypsin (Trypsin 0.05% / EDTA 1X, 25300-062, China), DPBS (Calcium, Magnesium free, 14200075, China). Epilife (30μM Calcium, MEPI500CA, China). Antibiotic-Antifungal Triple Antibiotic Solution 100X (Basal media, S120JV, China).

2.2 JMB326 information

The bacterial strain was isolated from the Himalayan region of Tibet, China. After cultivation and purification, it was identified as *L. reuteri* by 16s rDNA Sequencing and deposited at the China Center for Type Culture Collection (CCTCC) under the patent preservation name *L. reuteri* JMB326 on December 10, 2024.

2.3 LrFF-JMB326 preparation

For preparation, the strain was inoculated at 1% (v/v) into MRS broth and anaerobically cultured at 37°C for 16 h to obtain the seed culture. Subsequently, the seed culture was transferred at 5% (v/v) inoculation volume into YPD medium and subjected to anaerobic fermentation at 37°C for 48 h. The resulting fermentation broth was centrifuged (8000 rpm, 30min, 4°C) and filtered (0.22 μm) to obtain the JMB326 fermentation filtrate. The solid content of the filtrate was determined to be 10 mg/mL using the dry weight loss method. All samples were aliquoted and stored at -20°C for subsequent experiments.

2.4 Cell culturing

In this research, NHDF was used from passage 5 to passage 10, NHEK was used from passage 1 to passage 3, HaCAT was used from passage 6 to passage 15. NHDF was incubated in a CO₂ incubator, by using DMEM with 10% BCS and 1% Antibiotic-Antifungal Triple Antibiotic Solution. NHEK was incubated in a CO₂ incubator by using Epilife with Supplement S7 and 1% Antibiotic-Antifungal Triple Antibiotic Solution. HaCAT was incubated in a CO₂

incubator by using DMEM with 10% FBS and 1% Antibiotic-Antifungal Triple Antibiotic Solution. The medium for each cell was changed into a fresh medium within 48 hours. Cells are detached by 0.05% Trypsin when they are at 90% convergence.

2.5 Cell viability assay

NHDF and HaCAT were cultured in 96 well plates at 5×10^3 each well, while NHEK was cultured in 96 well plates at 8×10^3 each well. Cells were incubated in medium for 24 hours till they were fully attached to the bottom of 96 well plates. After culturing, cells were rinsed by DPBS twice, then treated the cells with UVA or UVB irradiation. NHEK and HaCAT were treated by UVB with 20 mJ/cm^2 , while NHDF was treated by UVA with 2.5 J/cm^2 . After treatment, cells were rinsed by DPBS twice.

For NHDF and HaCAT, the plates were divided into the non-treated group (NT), positive control (BCS/FBS), and testing group. For NHEK, the plates were only divided into NT and testing groups. Medium without serum was used for NT and the testing group, while positive control contained 10% of serum. The testing group was prepared with LrFF-JMB326 varying from 0.5% (v/v), 1% (v/v), 5% (v/v), 10% (v/v). The medium was given to each well, checking the cell condition by microscope whether cells were detached or not. Cells were incubated in a CO_2 incubator for 12 hours, and $10 \mu\text{L}$ CCK8 solution was added to each well and incubated for another 1 hour. Assay at 450 nm with a microplate reader.

2.6 Detection of intracellular ROS

NHDF was cultured in 12 well plates at 5×10^4 each well and separated into 2 groups which were NT and testing group. Medium without serum was used for NT and the testing group. 1% (v/v), 5% (v/v), 10% (v/v) of LrFF-JMB326 were used to testing group. Medium above was added after NHDF spread till 80% convergence.

NHDF was incubated in a CO_2 incubator overnight. 5 J/cm^2 UVA was used to stimulate NHDF to secrete ROS. ROS abundance was detected in NHDF with the final concentration at $5 \mu\text{M}$ (Dilution 1:500 in DMEM serum-free). NHDF was incubated in a CO_2 incubator for another 3 hours so that the probe could fully bind to ROS in the cell. Rinsing the cell with DPBS softly 3 times.

Pictures were snapped by an inverted fluorescence microscope (CKX53) at the fluorescence channel of GFP.

2.7 Mitochondrial membrane potential detection (JC-1)

NHDF was cultured in 12 well plates at 5×10^4 each well and separated into 3 groups which were NT, positive control (Carbonyl Cyanide3-ChloroPhenylhydrazone, CCCP), and testing group. Medium without serum was used for NT and the testing group, while positive control contained 10% of serum. 1% (v/v), 5% (v/v), 10% (v/v) of LrFF-JMB326 were used to testing group.

NHDF was incubated in a CO_2 incubator till 80% convergence. 5 J/cm^2 UVA was used to give pressure to the mitochondria of NHDF, which means to disorder the capability of mitochondria. Medium above was added after being treated by UVA, keeping the cell in a CO_2 incubator overnight.

A mitochondrial membrane potential detection Kit was used for detection. Rinsing the cell with DPBS twice to remove the impurity. CCCP was used as a positive control because CCCP can down-regulate the expression of high membrane potential at the final concentration of $10 \mu\text{M}$. Cells were incubated at 37°C CO_2 incubator for 20 minutes after adding JC-1 working buffer. Rinsing the cells with JC-1 washing buffer twice. Removing the DPBS and giving fresh DMEM to the well.

Pictures were snapped by an inverted fluorescence microscope (CKX53) at the fluorescence channel of GFP and FITC.

2.8 Immunofluorescence assay (IFA)

NHDF and HaCAT were cultured in 24 well plates at 2×10^4 each well and separated into 3 groups which are NT, positive control (Serum), and testing group. Medium without serum was used for NT and the testing group, while positive control contained 10% of serum. NHEK was cultured in 24 well plates at 5×10^4 each well and separated into 2 groups which were NT and testing group. 5% (v/v), 10% (v/v) of LrFF-JMB326 were used for testing group.

After culturing, cells were rinsed by DPBS twice, then treating the cells with UVA or UVB irradiation at 90% convergence of the cells. NHEK and HaCAT were treated by UVB with 20 mJ/cm², while NHDF was treated by UVA with 2.5 J/cm² or 5 J/cm² (The induction conditions were changed according to different markers). After treatment, cells were rinsed by DPBS twice. The medium above was added after being treated by UV irradiation. Cells were incubated in a CO₂ incubator overnight, rinsing the cells with DPBS twice to wash away the dead cells and impurities.

Cells were fixed by 10% formaldehyde-0.05% Triton X100 solution for 10 minutes. Rinsing the cells with DPBS 3 times. Cells were blocked by blocking buffer at room temperature (RT) for 1 hour. Adding primary antibodies and keeping it overnight. Rinsing the cells with DPBS 3 times. Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, and Donkey Anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 were used for secondary antibodies with the dilution of 1:1000. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus with the dilution of 1:1000. Incubating the cells after adding DAPI and secondary antibodies avoiding the light at RT for 1 hour. Rinsing the cell with DPBS 3 times.

Pictures were snapped by standing fluorescence microscope at the fluorescence channel of GFP.

2.9 Picture analysis and data statistics

All pictures snapped by microscopes were analyzed by ImageJ. For all data, the statistical significance was assessed by the *t*-test. In the statistical results, * referred to the *p* <0.05. compared with NT, ** referred to the *p*<0.01 and *** referred to the *p*<0.001.

3. Results

3.1 LrFF-JMB326 helps cells survive from Ultraviolet

Ultraviolet has negative effects on skin cells. As Figure 1 a~c shows, after being irradiated by 2.5 J/cm² UVA, NHDF treated with LrFF-JMB326 showed higher viability compared with NT. LrFF-JMB326 showed extraordinary effects on NHEK and HaCAT to recover from 20 mJ/cm² UVB.

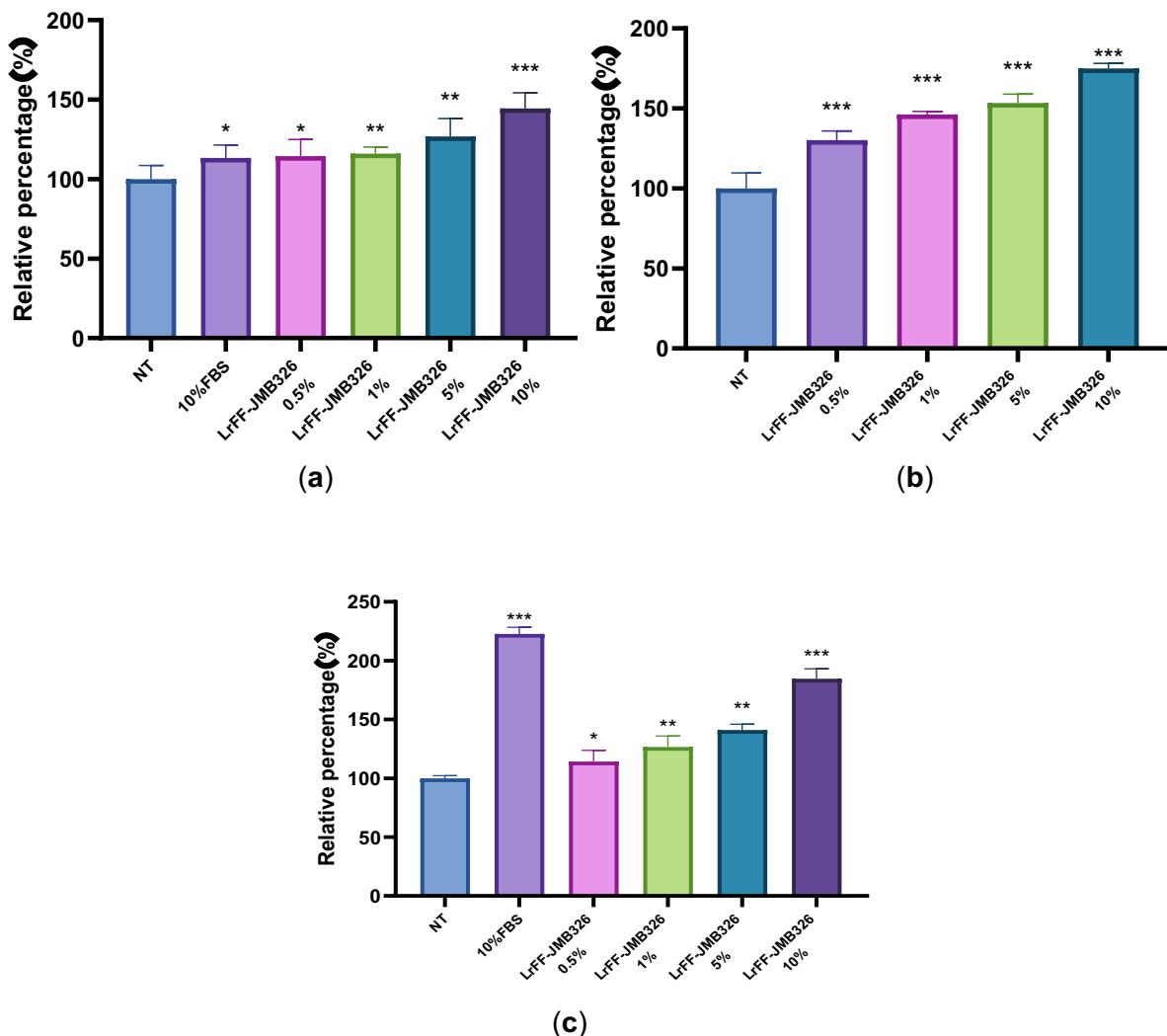
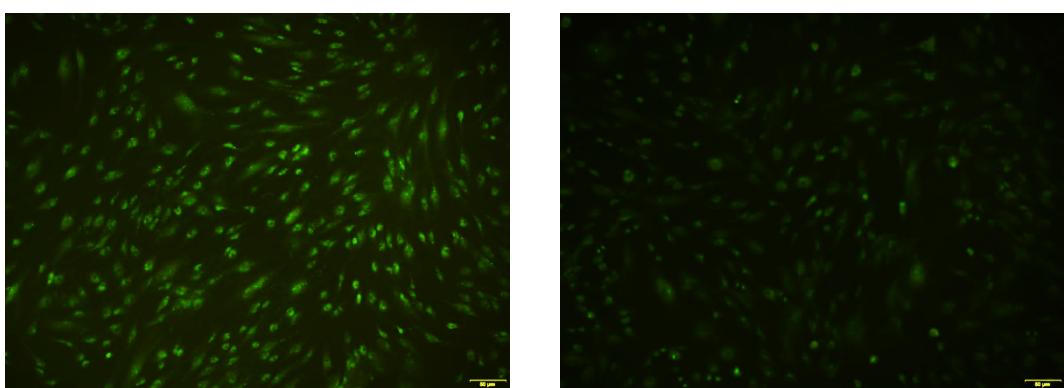


Figure 1. Cell viability results after treated by UV (a). NHDF viability results after treated by 2.5 J/cm² UVA. (b). NHEK viability results after treated by 20 mJ/cm² UVB. (c). HaCAT viability results after treated by 20 mJ/cm² UVB.

3.2 LrFF-JMB326 protects cells from ROS and enhances mitochondrial homeostasis

The production and content of ROS and the steady state of mitochondrial membrane potential can directly reflect the repair and survival ability of cells exposed to ultraviolet radiation. ROS, Mitochondrial membrane potential (JC-1) and DNA damage (γ -H2AX) were detected to evaluate how much LrFF-JMB326 could make NHDF alive after ultraviolet treatment.



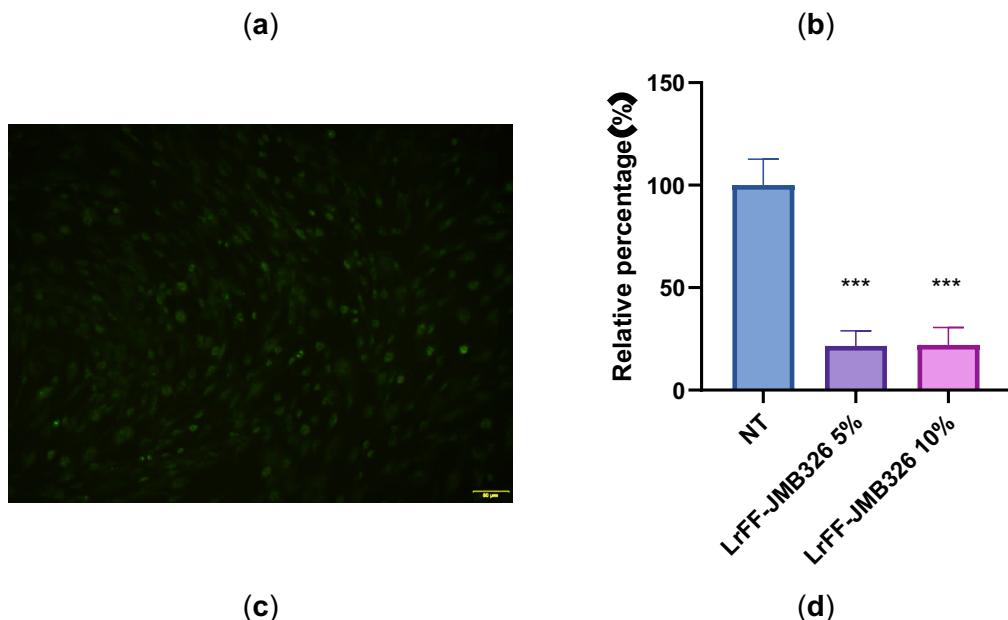


Figure 2. The ROS results of NHDF treated by 5 J/cm^2 UVA. ROS was detected by Green signal kit. (a). None treated group (NT, Magnification 10X). (b). LrFF-JMB326 5% (v/v) (Magnification 10X). (c). LrFF-JMB326 10% (v/v) (Magnification 10X). (d). Relative percentage of fluorescence intensity.

Compared with NT, LrFF-JMB326 relief NHDF from high pressure of ROS (Figure 2 a~c), ROS decreased 78.4% at 5% (v/v) and 77.9% at 10% (v/v), as Figure 2 d. shown.

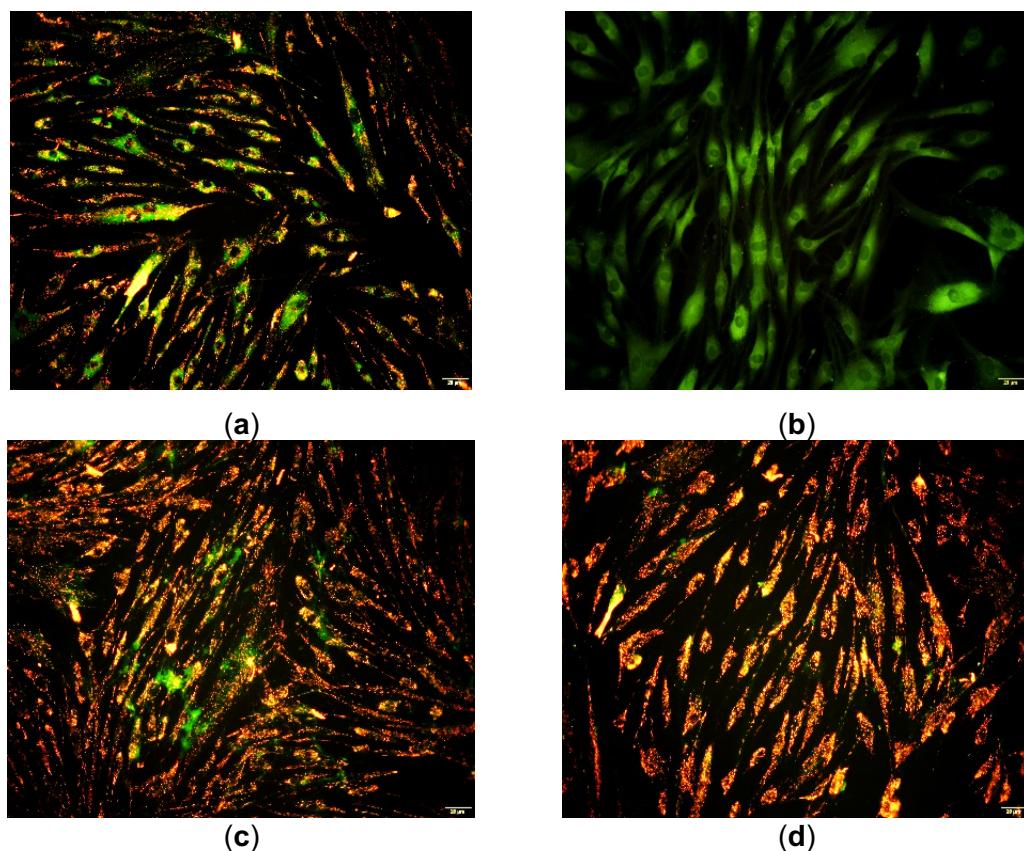


Figure 3. The JC-1 results of NHDF treated by 5 J/cm² UVA. JC-1 was detected by Mitochondrial membrane potential detection Kit. Red signals showed the condition when mitochondria in a high potential. Green signals showed the condition when mitochondria in a low potential. (a). None treated group (NT, Magnification 20X). (b). Positive control treated with 10 µM CCCP (Magnification 20X). (c). LrFF-JMB326 5% (v/v) (Magnification 20X). (d). LrFF-JMB326 10% (v/v) (Magnification 20X).

As is shown in Figure 3, after being treated by 5 J/cm² UVA, the potential of mitochondria became low with the green signals appeared (Figure 3 a). CCCP was used as a positive control to ensure NHDF can give the correct feedback when suffering from UVA or cured by LrFF-JMB326. CCCP can lower the mitochondrial membrane potential with the final concentration of 10 µM (Figure 3 b). After being treated with different concentrations of LrFF-JMB326, the high potential of mitochondria was enhanced and the low potential of mitochondria was decreased. It was shown dose-dependent that 10% (v/v) of LrFF-JMB326 had the best performance to enhance the mitochondria membrane potential (Figure 3 d).

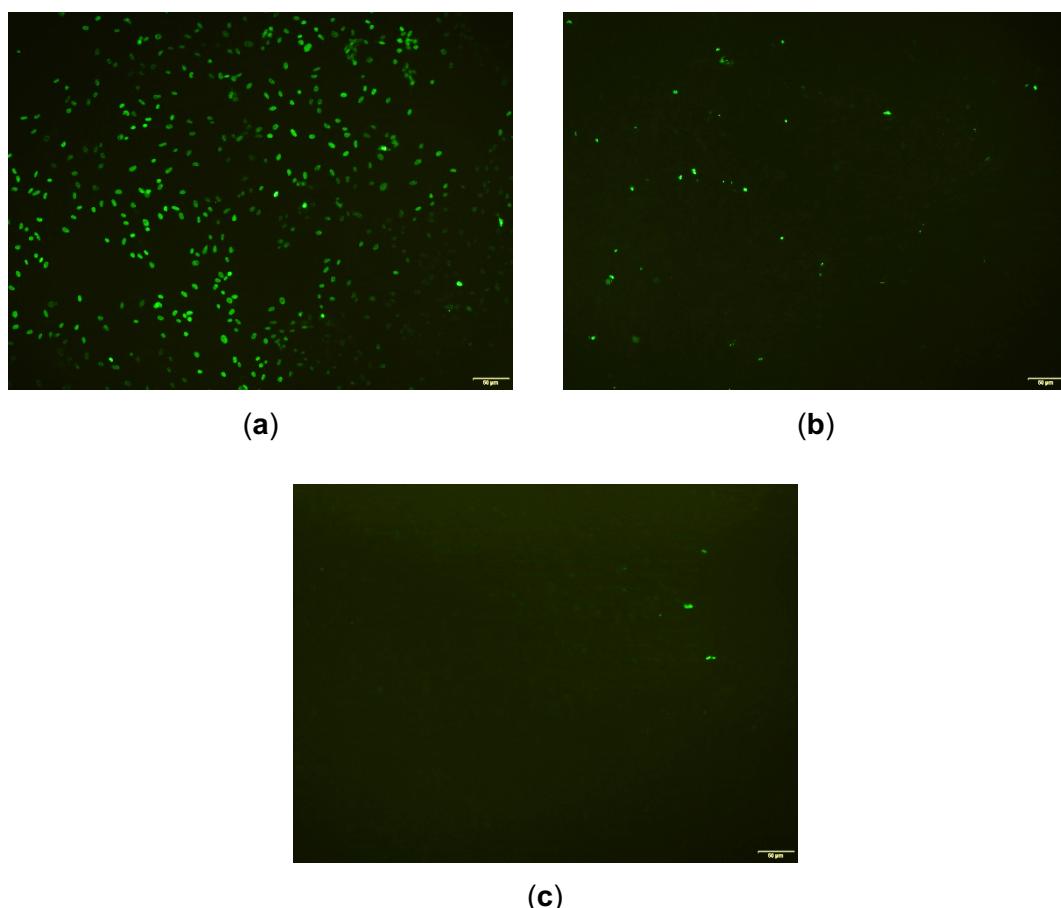


Figure 4. The γ-H2AX results of NHDF treated by 5 J/cm² UVA. γ-H2AX was detected by DNA damage detecting Kit, γ-H2AX of Immunofluorescence assay (Green signals). (A). None treated group (NT, Magnification 10X). (B). LrFF-JMB326 5% (v/v) (Magnification 10X). (C). LrFF-JMB326 10% (v/v) (Magnification 10X).

γ-H2AX usually expresses when cells are attacked by Ultraviolet and H₂O₂. However, γ-H2AX is a protein for recovery, it is always regarded as the extent of damage to the DNA inside cells. As Figure 4 shows, NT which was treated by 5 J/cm² UVA without rescue

showed a high expression of γ-H2AX. LrFF-JMB326 can protect DNA in cells from UVA, as γ-H2AX is expressed lower than NT.

4. Discussion and Conclusion

Himalaya wild probiotic strains and fermentation-derived ingredients show impressive active capacities in skincare. In this study, immunofluorescence assay and CCK8 were used to show the capability of LrFF-JMB326 which enables NHDF, NHEK and HaCAT to survive from Ultraviolet. We found that LrFF-JMB326 significantly reduced the accumulation of intracellular ROS by 78.4% (*** at 5% (v/v) and 77.9% (*** at 10% (v/v). LrFF-JMB326 shows a high potential to protect the cell. Therefore, the decrease of intracellular reactive oxygen species is inevitable. When intracellular ROS is maintained at normal levels, mitochondria are healthy and their function is not affected. At the same time, resistance to UV damage to DNA in cells is also one of the important effects of LrFF-JMB326. These advantages make normal skin cells more tolerant to UV rays.

5. Reference

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