Innovative screening and application of *Lactobacillus paracasei* with antioxidant efficacy

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ABSTRACT

Background: In recent years, probiotic skincare products have been gained extensive attention and application. In order to make up for the shortcomings of the current research methods in probiotic skincare products, we have established an efficacy-oriented strategy for screening and developing strains with specific antioxidant activity.

Methods: With efficacy-oriented way, the UVB-H_2O_2 dual-model was established for preliminary screening, further 4 rounds of screening and verification were carried out by multiple dimensions. Fortunately, a target strain with strong antioxidant effect, *Lactobacillus paracasei*, was obtained from 85 original probiotic strains, as well, the *Lactobacillus* Ferment (LF) product with better stability was prepared.

And then, the safety of this LF product was evaluated out by cytotoxicity test with keratinocyte (HaCaT) cells -MTT model, ocular irritation test by Chorioallantoic Membrane Vasular Assay (HET-CAM), as well, human patch test was performed on 30 subjects.

Results: It had been showed that the LF product was safe as a cosmetic raw material by the results of the safety evaluation test. Compared with the H_2O_2 injury group, the T-AOC, SOD activity, CAT activity and Nrf2 secretion were significantly improved in 5% LF product supplement group. Meanwhile, the LF product decreased the ROS level. *In-vivo* test, skin

moisture content and elasticity of subjects were improved, combined with reduction of the area of brown spots.

Conclusion: It is verified that establishment of specific screening models can effectively obtain functional probiotic strains, and the targeted screening strategy will provide a new method for the development of probiotic skin care products or active ingredients.

Key words: *Lactobacillus* ferment, Efficacy-oriented screening, Antioxidant, Anti-aging, Personal care product.

1. **Introduction**.

Probiotics are live microorganisms that are beneficial for the health of the host, as well for the skin. With the deepening and development of research, more beneficial functions of probiotics have been discovered, such as improving intestinal diseases [1], central nervous system diseases [2], and anti-tumor [3]. In recent years, with the in-depth study of skin microecology, probiotic skincare products have gained extensive attention due to their mild, natural, efficient and other characteristics. At present, the probiotic skincare products play important roles in anti-photoaging [4] [5], anti-oxidation [6], treatment of atopic dermatitis [7], barrier damage repair [8] and so on.

However, most of the probiotic products are embodied reflect good concepts rather than substantive efficacy. Meanwhile, the efficacy claims are confusing, lack of adequate experimental data support, and the direction of development is not clear. In order to make up for the shortcomings of the current research methods in probiotic skincare products, we have established an efficacy-oriented strategy for screening and developing strains with specific antioxidant activity.

2. Materials and Methods

2.1 Screening of probiotics with antioxidant function

Human immortalized keratinocyte (HaCaT) cell was purchase from China Center For Type Culture Collection. Firstly, the ultraviolet radiation B (UVB) cell damage model and the hydrogen peroxide (H_2O_2) cell damage model were established, respectively. and the antioxidant efficacy of the sample was evaluated by the cell survival rate. 85 probiotics strains were selected from our library of strains for testing. Then, the fermentation filtrate or

lysate of the 85 strains were prepared for further testing by the UVB-H_2O_2 dual-model screening technology. Subsequently 10 probiotic strains were obtained, then the H_2O_2 cell damage model was re-used, and the antioxidant ability was as the evaluation indicator.

The efficacy of strains was verified from multiple dimensions such as total antioxidant capacity (T-AOC), superoxide dismutase (SOD) enzyme activity, reactive oxygen species (ROS) level and Nrf2 secretion. Among them, the intracellular ROS level was detected by a fluorescence spectrophotometer, T-AOC and the ROS level was expressed by the relative fluorescence intensity of DCFH-DA. The T-AOC and SOD enzymes were detected by T-AOC and superoxide dismutase (SOD) detection kit (Nanjing SenBeiJia Biological Technology Co., Ltd.) respectively. BCA protein concentration assay kit (Beyotime Biotechnology P0009) and ELISA assay kit (SenBeiJia Biological Technology Co., Ltd.) were used to detect Nrf2 secretion severally. The culture conditions used in the negative control (NC) were normal culture medium without induction; the culture conditions used in the test sample (TA) to be tested were culture medium containing 5% of the sample, and the culture conditions used in the positive control (PC) were culture medium containing 0.04% of nicotinamide. Induction conditions were 50 μmol/L H_2O_2 Reagent.

2.2 Safety evaluation

2.2.1 Cytotoxicity test

The logarithmic growth phase human epidermal keratinocytes HaCaT cells in logarithmic growth phase were inoculated in a 96-well plate, and the culture system was DMEM high-glucose medium supplemented with 10% fetal bovine serum. Samples (LF) were prepared with serum-free culture medium with concentrations of 0.5%, 1.0%, 2.5%, 5.0%, and 10.0%, respectively, and sterilized by filtration. After the inoculated cells were routinely cultured for 24 h, the culture medium was discarded and replaced with 100 μL of samples, and the serum-free culture medium was used as a negative control (NC). Three parallel wells were set up for each concentration. After culturing for 24 h, the relative growth rate (RGR) of cells was detected by CCK-8 method. The RGR was the ratio of the absorbance of the sample group to that of the negative control group. When the RGR is lower than 70%, the sample is considered to have potential cytotoxicity.

2.2.2 Ocular irritation

Ocular irritation experiments were carried out using chicken embryo CAM at the 10^{th} days age after fertilization purchased from Beijing Boehringer Ingelheim Biotech Co., Ltd.

The treatment method of the Reference Material [IR, the reagents used are Texapon ASV (0.5%, 1%, 5%), NaOH (0.2%, 0.3%, 0.5%)] is as follows: The internal reference substance was added 300 μL directly on the surface of CAM, at least 2 eggs at each concentration. Tining for 5 min, the substance was gently rinsed with normal saline. The following three endpoints: bleeding, vascular melting and coagulation were observed at 30 s and the degree of reaction on these three endpoints were scored.

Test samples (TA, LF dilution) and control substances (positive control PC: 0.9% NaCl solution; negative control NC: 1% SLS solution) were treated as follows: The samples and control substance were added 300 μL on the surface of CAM (6 eggs) respectively. Tining for 3 min, the test sample was gently rinsed with 0.9% NaCl. The following three endpoints: bleeding, vascular melting and coagulation were observed at 30 s and the degree of reaction on these three endpoints were scored.

The severity of the test samples was assessed using the S-Score (severity score) model in the tests according to the endpoint evaluation method. The reaction degree of the test samples was compared with that of the internal reference substance to determine the score (Table 1), and the range is 0~3 points (0 = no reaction, 1 = slight reaction, 2 = moderate reaction, 3 = severe reaction). The scores at the same endpoint of all the repeated eggs of each test sample were summed to obtain the S-Score of the three endpoint reactions separately. The highest S-Score in the three end-point reactions was taken as the final score of the test sample. The results were assessed according to the S-Score in irritancy classification ($S<6$: no irritation; $6 \leqslant S \leqslant 15$: moderate irritation; $S \geqslant 15$: severe irritation).

Table 1 End point scoring method

Score	0	1	2	3
Hemorrhage	No hemorrhage	Weak: only fine blood vessels are bleeding and only small amounts of blood are flowing out (e.g. Texapon ASV, active substance content (AS) 0.5%, 5 min. contact)	Moderate: fine blood vessels and large vessels are bleeding and distinct amount of blood are flowing out (e.g. Texapon ASV, 1% AS, 5 min. contact)	Strong: nearly all blood vessels are bleeding with a considerable amount of blood flowing out (e.g. Texapon ASV, 5% AS, 5 min. contact)
Lysis	No lysis	Weak: only small blood vessels are lysing (e.g. Texapon ASV, 0.5% AS, 5 min. contact)	Moderate: small and large blood vessels are lysing (e.g. Texapon ASV, 1% AS, 5 min. contact)	Strong: large blood vessels and whole cords are lysing (e.g. Texapon ASV, 5% AS, 5 min. contact)
Coagulation	No coagulation	Weak: only weak intravascular and/or extravascular coagulation in CAM, or/and light opacity is observable (for coagulation, e.g. 0.2% w/w NaOH, 5 min)	Moderate: moderate extravascular coagulation in CAM, or/and moderate opacity is observable (for coagulation, e.g. 0.3% w/w NaOH, 5 min)	Strong: severe distinct extravascular coagulation in CAM, or/and opacity is observable (for coagulation, e.g. 0.5% w/w NaOH, 5 min)

2.2.3 Human patch test

Thirty healthy volunteers from 18 to 60 years old who met the test requirements were recruited. The patch test was performed using an 8 mm Finn Chamber patch device. The patch test process was as follows:

LF samples were diluted with purified water to 100%, 50%, 10% and 2.5%, respectively, and purified water was used as control. 0.020 mL of the sample was put into the patch tester. The tester was taped to the flexible forearm for 24 h.

The cutaneous reaction was observed after removing the tester for 30 min. If the cutaneous reaction is negative, then observe the cutaneous reaction after removing the tester for 24 h and 48 h respectively. The results were recorded according to the following standard (Rating Leval & Skin reaction): 0 = Negative; 1 = Weak erythema, Xerosis cutis, Wrinkle; 2 = Erythema, Edema, Papula, Wheal, Desquamation, Cracks; 3 = Obvious erythema, Edema,

Phlycten.

2.3 Efficacy evaluation

2.3.1 Cell efficacy test (*in vitro*)

Human immortalized keratinocyte HaCaT cells (China Type Culture Collection Center) were used to measure the antioxidant efficacy of samples from multiple dimensions such as T-AOC, SOD enzyme activity, ROS value, catalase (CAT) and Nrf2 secretion. The methods and kits used are the same as these in Section 2.1, CAT was detected by ELISA assay kit (SenBeiJia Biological Technology Co., Ltd.). The experimental groups and conditions are shown in Table 2.

Table 2 experimental groups and conditions

Group	Culture conditions	Induction condition
Negative control (NC)	Culture medium	-
Model control (M)	Culture medium	50 μmol/L H_2O_2
Test sample (TA)	Culture medium containing different concentrations of samples (0.5%、2.5%、5%)	50 μmol/L H_2O_2
Positive control (PC）	Culture Medium containing 25 μg/mL vitamin C	50 μmol/L H_2O_2

2.3.2 LF Efficacy evaluation（*in-vivo* test）

Fifteen healthy subjects aged 20-45 were recruited for LF efficacy evaluation. After facial cleaning for 15 min, the initial values of stratum corneum water content (Corneometer CM 825 Courage + Khazaka), skin elasticity (German multi-probe skin testing system MPA580 Courage + Khazaka) and brown spot area (German VISIA CR facial image analysis system) were measured in the left and right half of the face. Then the same amount of 2.5% LF essence and placebo (essence without LF) were used on the different side of face in the morning and evening, and all the indicators were checked again at 7^{th}, 14^{th} and 21^{st} day after the first use.

3. Results

3.1 Screening of probiotics with antioxidant function

3.1.1 UVB-H_2O_2 dual model screening results

The screening results of probiotics (85 strains) fermentation filtrates based on the UVB-H_2O_2 dual-model screening technology were shown in Figure 1. *Lactobacillus paracasei* G4, *Lactobacillus casei* G5, *Lactobacillus plantarum* Z3, *Lactobacillus plantarum* Z4,

Lactobacillus plantarum Z5, *Bifidobacterium animalis* DW1, *Bifidobacterium animalis* DW2, *Bifidobacterium animalis* DW3, *Bifidobacterium animalis* DW4, *Bifidobacterium animalis* DW5 showed potential antioxidant effects, and the fermentation filtrate and its lysis of the above 10 strains for further verification.

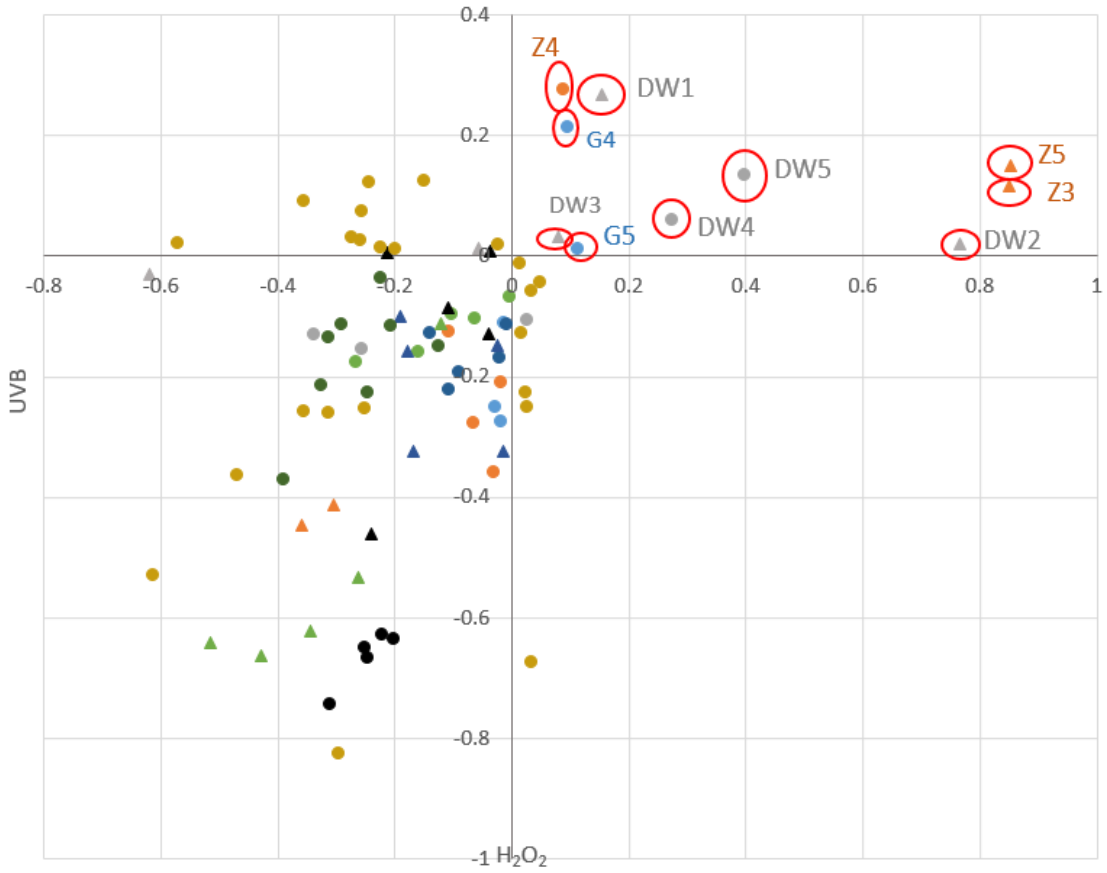

Fig.1 Screening strains by UVB-H_2O_2 dual-model technology

3.1.2 Repeated validation of screening strains

The 10 strains screened out were further tested, and the results were shown in Figure 2. Among the fermented filtrates and lycogenesis of 10 strains, the fermented filtrates of *Lactobacillus paracasei* G4, *Lactobacillus casei G5*, *Lactobacillus plantarum* Z4, *Lactobacillus plantarum* Z5, *Bifidobacterium animalis* DW1, *Bifidobacterium animalis* DW2, and lysate of *Lactobacillus casei* G5 showed potential antioxidant properties. Therefore, these above 6 strains (7 fermentation filtrates/ lysates) were selected for further antioxidant efficacy studies.

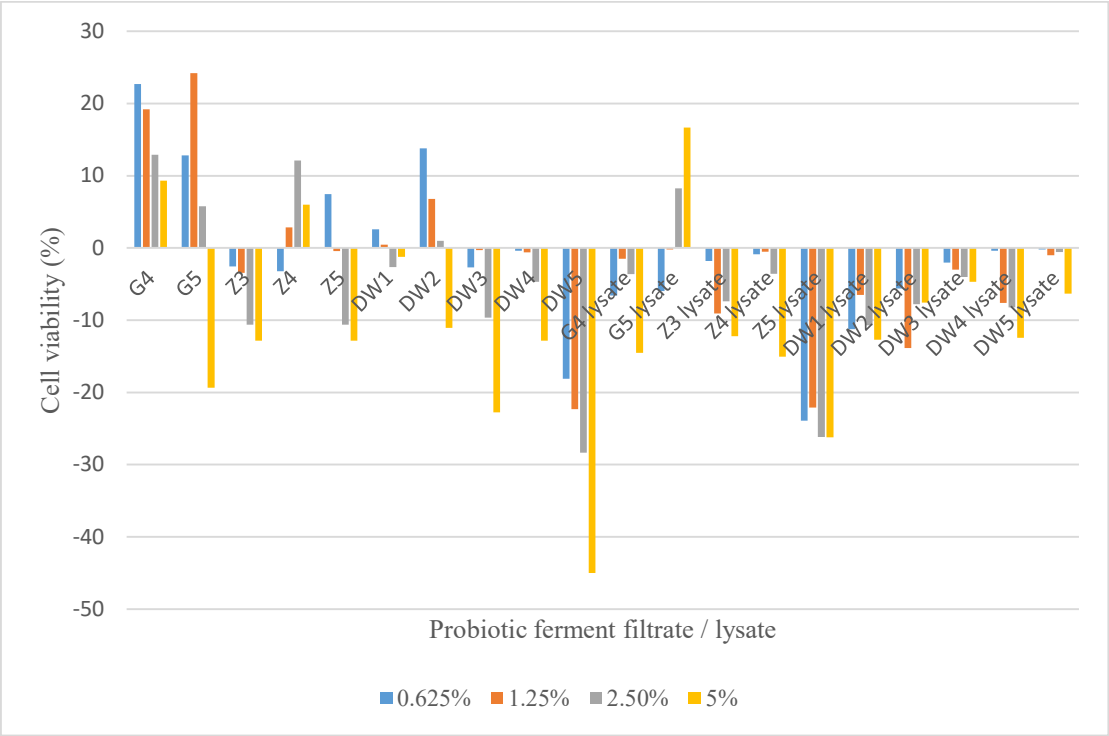

Fig.2 Repeat validation of ferment filtrate/ lysate - compared with model group

3.1.3 Antioxidant efficacy test and strain screening

The antioxidant efficacy of the selected probiotic fermentation filtrate / lysates was detected, and the positive control group with nicotinamide was used as the evaluation standard to evaluate the antioxidant effect. Seven kinds of fermentation filtrates/ lysates obtained by repeated verification were tested for T-AOC and SOD activity. As shown in Figure 3a, the scavenging effects of the fermentation filtrates of *Bifidobacterium animalis* DW1, *Bifidobacterium animalis* DW2, and *Lactobacillus paracasei* G4 on ABTS and free radicals were higher than those of the negative control (NC), and similar to that of the PC group. As shown in Figure 3b, after treated with the LF for 24 h, the SOD enzyme activities of the filtrates in *Bifidobacterium animalis* DW1, *Bifidobacterium animalis* DW2, *Lactobacillus paracasei* G4, *Lactobacillus casei* G5, *Lactobacillus plantarum* Z4, *Lactobacillus plantarum Z5* groups were 5.48, 4.84, 3.91, 2.24, 2.50, 3.59 U/mg respectively, and the enzyme activity of lysate in *Lactobacillus casei* G5 group was 2.28 U/mg. Meanwhile, the SOD enzyme activity in the negative control group was 2.63 U/mg. Compared with the NC group, the SOD enzyme activities of fermentation filtrate in *Bifidobacterium animalis* DW1, *Bifidobacterium animalis* DW2 and *Lactobacillus casei* G4 were increased by

108.42%, 83.97% and 48.95%, respectively, which revealed better antioxidant effect. Besides, the SOD enzyme activities of DW1 and DW2 were higher than that of the PC group, and the SOD enzyme activities of G4 was similar to that of the PC group. Therefore, the fermentation filtrates of these strains were selected for further efficacy verification.

The fermentation filtrate prepared by the above three strains was used for the determination of ROS level. As shown in Figure 3c, compared with the positive control (PC), the ROS levels of the fermentation filtrates in *Bifidobacterium animalis* DW2 and *Lactobacillus paracasei* G4 groups were significantly decreased ($p < 0.01$), and similar result was obeserved in the NC group.

Due to H_2O_2 in the cell culture medium, Nrf2 signal pathway was activated in the PC group, *Bifidobacterium animalis* DW2 and *Lactobacillus paracasei* G4 (Figure 3d). Compared with the NC group, nicotinamide, DW2 and G4 significantly enhanced the secretion of Nrf2 ($p <0.05$), while DW1 did not contribute to promoting the secretion of Nrf2. These results indicated that the fermentation filtrate of *Bifidobacterium animalis* DW2 and *Lactobacillus paracasei* G4 possessed the potential to activate the Nrf2 secretion pathway againstthe oxidative damage caused by H_2O_2.

Based on the results of ROS and Nrf2/HO-1 signaling pathway-related cytokine secretion experiments, it was shown that the fermentation filtrate of *Bifidobacterium animalis* DW2 and *Lactobacillus paracasei* G4 strains screened from the initial three samples had obvious antioxidant properties. In addition, the cell viability of G4 group was higher than that of DW2 group (Figure 2). Therefore, *Lactobacillus paracasei* G4 was subsequently selected to prepare LF for further study.

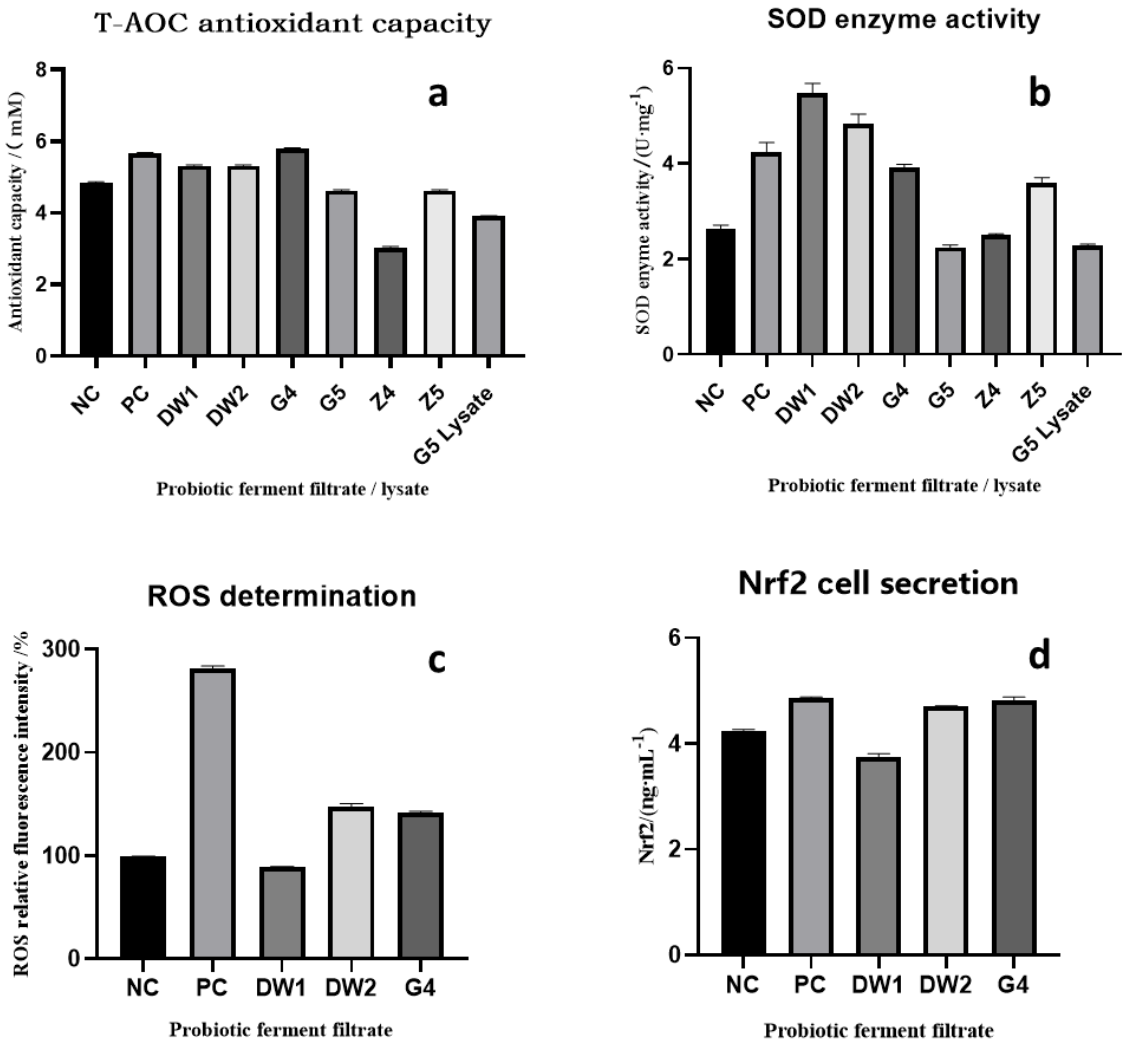

Fig 3 Study The verifed results on antioxidant efficacy-oriented strategy for screening motivated strain

3.2 Safety testing of *Lactobacillus* Ferment (LF)

3.2.1 Cytotoxicity tests of *Lactobacillus* Ferment (LF)

The results of cytotoxicity test were shown in Table 3. The results of RGR in LF of *L. paracasei* G4 groups were higher than 70%, which determined that LF of *L. paracasei* G4 had no obvious cytotoxicity in the concentration range of 0.5% to 10.0%. And in the concentration range of 5.0% - 10.0%, LF can significantly promote the proliferation of HaCaT cells.

Table 3 The RGR of HaCaT cells treated by LF

	Control	0.5%	1.0%	2.5%	5.0%	10.0%
LF	100.00±8.23	82.17±11.25*	85.55±4.89*	107.29±10.77	129.94±5.53***	110.30±5.81 *

Note: *: p< 0.05 compared with Control; **: p < 0.01 compared with Control; ***: p < 0.001 compared with Control;

3.2.2 The results of ocular irritation test

As shown in Table 4, according to the judgment criteria of HET-CAM (Endpoint Scoring Method), the result of the test sample "90% LF" was 4, which showed non-irritant effect.

Table 4 The result of Ocular irritation test

Negative Control (NC) -0.9% NaCl	CAM#	Hemorrhage	Lysis	Coagulation
	1-6	0	0	0
	Scores	0	0	0
	S-Scores	0		
Positive Control (PC) -1% SLS	CAM#	Hemorrhage	Lysis	Coagulation
	1	2	3	2
	2-3	1	2	2
	4-5	1	3	2
	6	1	3	2
	Scores	7	16	12
	S-Scores	16		
Test Sample (TA) -90% LF	CAM#	Hemorrhage	Lysis	Coagulation
	1	1	0	0
	2-3	0	0	0
	4-6	1	0	0
	Scores	4	0	0
	S-Scores	4		

3.2.3 Human patch test

After removing the tester for 30 min, skin reaction was observed at 24 h and 48 h. Results: 100%, 50%, 10%, 2.5% concentration after the subject uses the sample showed negative reactions, as shown in Table 5.

Table 5 Test result of Human patch test

Rating level	2.5% LF	10% LF	50% LF	100% LF	Purified water
0	30	30	30	30	30
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0

3.3 Results of LF Efficacy Assay

3.3.1 The results of efficacy evaluation *in vitro*

According to the previous qualitative experimental results, it was concluded that LF could improve the antioxidant capacity of cells. Based on the quantitative analysis of high, medium and low concentrations of LF (Figure 4a), the content of T-AOC under the condition of 5% LF was the highest. Compared with the model group, the T-AOC capability of 5%, 2.5% and 0.5% LF supplement were enhanced by 2274.07%, 1167.90% and 393.83%, respectively ($p < 0.01$).

As shown in Figure 4b LF significantly increased the activity of SOD in cells. Compared with the model group, the results of SOD activity in 5%, 2.5% and 0.5% LF supplement group were increased by 483.58%, 430.29% and 324.45%, respectively ($p< 0.01$).

The results of ROS detection (Figure 4c) showed that the relative fluorescence intensity decreased by 37.79%, 41.16% and 54.90% respectively after 5%, 2.5% and 0.5% LF treatment, which could significantly improve the oxidative damage of HaCaT cells induced by H_2O_2 ($p < 0.01$).

As shown in Figure 4d, compared with the model group, CAT activity was increased by 3324.24%, 1024.24% and 736.36% after 5%, 2.5% and 0.5% LF treatment, respectively ($p <0.01$). The results showed that LF could resist the oxidative damage of HaCaT cells induced by H_2O_2 and had a certain protective effect on HaCaT cells from cytotoxicity caused by H_2O_2.

As shown in Figure 4e, compared with the model group, the secretion of Nrf2 was increased by 64.83%, 77.93% and 61.84% after treated with 5%, 2.5% and 0.5% LF, respectively, showing extremely significant difference ($p<0.01$). It is suggested that LF can promote the secretion of Nrf2 by HaCaT cells and improve the antioxidant stress ability of

skin.

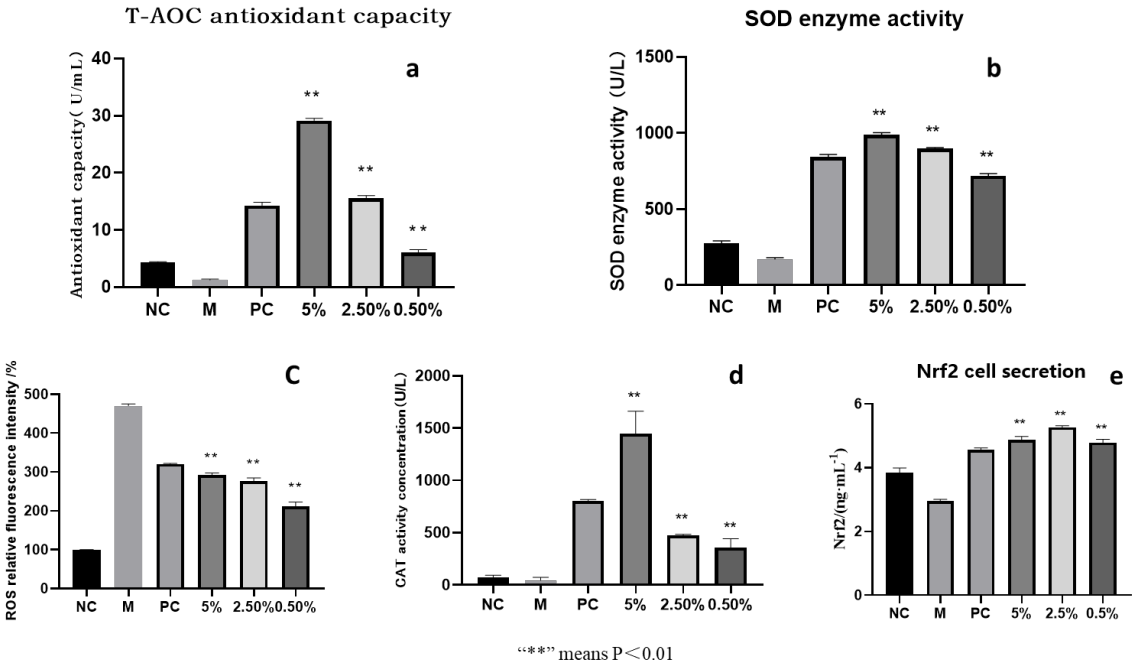

Fig. 4 The result of antioxidant efficacy evaluation

3.3.2 Results of efficacy evaluation *in-vivo*

As shown in Figure 5a, the initial moisture content of stratum corneum was set at 100%. Compared with the placebo, the moisture content of stratum corneum was significantly increased in the LF group after a 3-weeks intervention, which was higher than that in the placebo group (12%-14%).

As shown in Figure 5b, the initial value of skin elasticity was set to 100% before use. Compared with the placebo, the skin elasticity of LF was significantly improved within 3 weeks, especially after one-week intervention, which was 10% higher than the placebo.

As shown in Figure 5c, the proportion of brown spot area in the fixed selection before use was set to 100%. Compared with the placebo, the use of LF essence significantly reduced the area of brown spot area within 3 weeks (reducing 10%-12%), especially at 7^{th} and 14^{th} day, which showed significant difference, indicating that LF can significantly reduce the skin pigmentation.

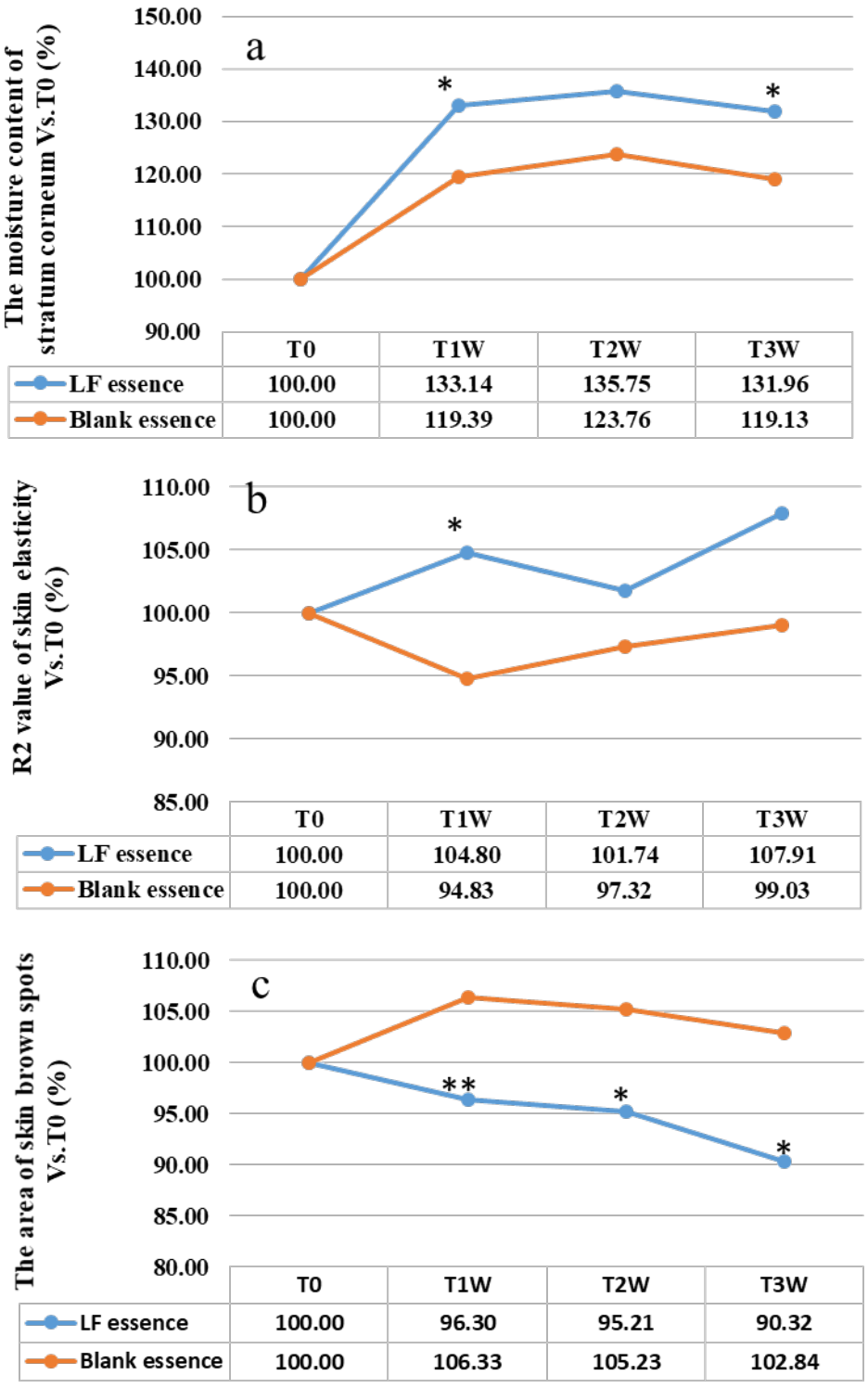

Fig. 5 Effect of LF on the moisture content of stratum corneum (a), skin elasticity (b), area of skin brown spots(c). ** , represents the comparison with the blank essence group（$p<0.01$） * , represents the comparison with the blank essence group ($p<0.05$).

According to the cytotoxicity assay, the viability of HaCaT cells maintain above 80% in the range of 0.5%-10%; On account of the HET-CAM model, 90% LF is non-irritating; Moreover, the results of patch test showed that 10% LF had no potential irritation and sensitization. Thus LF product, as cosmetic raw material, is safe for skincare industry.

Based on the HaCaT-H_2O_2 damage model, the T-AOC, SOD activity, CAT activity and Nrf2 secretion of 5% LF group were significantly improved compared with the H_2O_2 damage group. As well as the level of ROS was decreased significantly with 5% LF product. From these results, it was found that the Nrf2 antioxidant signal pathway was activated by LF, then skin antioxidant capacity was enhanced by the increasing ability of T-AOC, SOD and CAT. Additionally, based on the *in-vivo* efficacy evaluation, the skin water content was increased by 12% to 14% compared with the placebo within 3 weeks application, the skin elasticity was improved by 10% within one week, and the area of brown spots was reduced by 10% to 12% within three weeks.

Discussion.

Through efficacy-oriented screening strategy, probiotic skincare products with specific efficacy were obtained, which provided new ideas and innovative directions. Taking *Lactobacillus paracasei* as an example, we focused on the anti-oxidant efficacy and assessed the indicators on antioxidant signal pathways. Thus the *Lactobacillus* Ferment (LF) targeted on anti-oxidant and anti-aging was developed. The results showed that the *Lactobacillus* Ferment (LF) activated the Nrf2 antioxidant pathway, sequentially, enhanced the antioxidant capacity of T-AOC, SOD and CAT, as well, LF played a crucial part in reducing oxidative damage. Furthermore, *in-vivo* tests have showed that the LF product has been shown to firm and brighten skin by increasing skin hydration and skin elasticity, as well, reducing skin pigmentation.

Conclusion.

It is a new effective method for the development of probiotic skin care products or active ingredients that establishing a screening model to obtain targeted strains with specific efficacy. At present, the *Lactobacillus* Ferment product with specific antioxidant effect obtained by this method has a good application prospect in the field of skincare industry.

Conflict of Interest Statement. NONE.

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