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"The triple-fermentation essence empowers the journey of skin micro-ecological nourishment"

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1. Introduction

With the continuous upgrading of the cosmetics market, the development of biotechnology, and consumers' increasing concern for the quality, safety, and environmental protection of cosmetics, the era of biological skincare is gradually approaching. The use of microbial technology, fermentation technology, biomimetic technology, etc. in cosmetics research and development has become mainstream. Fermentation technology and microbial technology are usually complementary[1].Active substances derived from fermentation have multiple effects on the human body and are commonly used in the field of cosmetics, such as antioxidant, anti-inflammatory, antibacterial, moisturizing, whitening, anti-aging, etc[2].

In this study, the product contains three types of biological fermentation active substances, among which *BIFIDA FERMENT LYSATE* can improve skin damage, soothe irritation, maintain skin stability and anti-aging; *YEAST FERMENT EXTRACT* can accelerate skin metabolism, moisturize, brighten, and whiten the skin; *LACTOBACILLUS/OYMILK FERMENT FILTRATE* can enhance skin microbial diversity, promote health, and repair barriers. The three main fermentation ingredients are scientifically proportioned to achieve optimal results[3].

In the efficacy evaluation, 32 healthy adult volunteers were selected, we tested and analyzed various skin indicators of the subjects before and after using the product[4], as well as their subjective and self-evaluation[5]. The comprehensive results showed that this product can provide good nourishment to the subjects' skin without any adverse reactions. Combining 16S rDNA amplification sequencing technology, we found that this product plays a key role in regulating the balance of skin microbiota. It is worth mentioning that the use of this product can reduce the content of some harmful bacteria in the skin.

2. Materials and Methods

2.1 Materials and Equipment

Yeast nourishing essence, placebo, DNA extraction kit, PCR amplification reagent (Taq enzyme dNTPs、 Primers, agarose, nucleic acid dyes, DNA markers, Tewameter TM Hexx (Courage&Khazaka, Germany) Cutometer MPA580 (Courage&Khazaka, Germany), VisioScan VC20 Plus (Courage&Khazaka, Germany), centrifuge, PCR, gel electrophoresis, nucleic acid sequencing, pipette.

2.2 Human test

2.2.1 Selection of Test Objects

Select 32 healthy adults aged 18-60 with fine lines, dryness, and roughness on their faces; Can accept skin examination and pre-treatment in the testing area; Be able to understand the

experimental process, voluntarily participate in the experiment, and sign a written informed consent form.
Subjects with the following conditions were excluded:
(1) Pregnant or lactating women, or those who have recently planned to conceive;
(2) Highly sensitive constitution, with a history of allergic diseases and cosmetics allergies;
(3) Suffering from skin diseases such as psoriasis, eczema, atopic dermatitis, severe acne, or other chronic systemic diseases;
(4) There are birthmarks, pigmentation, inflammation, scars, pigmented nevi, hirsutism and other phenomena on the skin of the test site;
(5) Oral or topical use of anti-inflammatory drugs such as corticosteroids within the past month;
(6) Have used cosmetics or other products with similar efficacy in the past 2 weeks;
(7) Participated in human clinical trials of cosmetics within the past month;
(8) Other clinical evaluations suggest that it is not suitable to participate in the trial.
2.2.2 Testing Environment
The testing environment is at a temperature of 20°C~22°C and a humidity of 40%~60% RH, which meets the requirements of the scheme design.
2.2.3 Product usage method
(1) The subjects were designed to use the test product in the experimental area according to the experimental protocol.
(2) The subjects apply an appropriate amount evenly to their face in the morning and evening, and massage until absorbed. Cannot enter during the entire testing period
Long term sun exposure, outdoor activities, tourism, etc. are not allowed to use cosmetics or drugs with similar efficacy to the product, and subjects are not allowed to change their daily care habits.
2.2.4 Instrument testing indicators
(1) Transdermal water diversion loss
Measuring instrument: Tewameter TM Hex (Courage&Khazaka, Germany) for measuring skin surface moisture loss.
Measurement requirement: Take the average of 3 tests.
Parameter explanation: The lower the value, the less transdermal water loss. Company: g /（$h \cdot m^2$）。
(2) Skin elasticity R2 value
Measuring instrument: Cutometer MPA580 (Courage&Khazaka, Germany) for skin elasticity testing.
Measurement requirement: Take the average of 3 tests.
Parameter explanation: The higher the R2 value, the better the skin elasticity. Unit: Non dimensional.
(3) Smoothness parameter (SEsm)
Measuring instrument: VisioScan VC20 Plus (Courage&Khazaka, Germany).
Measurement requirements: Collect images once and analyze images once.
Parameter explanation: SEsm is a smoothness parameter, the lower the value, the smoother the skin. Unit: Non dimensional.
2.2.5 Subjective evaluation
The subjective evaluation criteria are shown in Table 1.

Table 1. Subjective Evaluation

Evaluation method	Assessment requirements		Evaluation items
Subjective assessment (VAS) Assessment - Nourishing	Three visual acuity assessors used Visual Analog Scale (VAS) to score the skin	0-10 points (The higher the score, the better the effect)	Skin delicacy

self-assessment	condition of the subjects, took the average, and recorded it Evaluate the user experience and skin condition of the product after 4 weeks of use by the subjects.	1-5 points	See self for details Evaluation results
Safety assessment	Record whether adverse reaction symptoms occur, the name of the symptoms, the severity of the symptoms, the start time, duration, and location of the symptoms.	Inquire and self describe	

2.2.6 Adverse Reaction Evaluation

The grading standards for adverse reactions are determined according to the grading standards for skin adverse reactions in human trial tests specified in the 2015 version of the "Technical Specification for Safety of Cosmetics", as detailed in Table 2; The severity grading criteria for adverse reactions are shown in Table 3

Table 2. Grading Standards for Skin Reactions in Human Trial Trials

Skin reactions	grade
No reaction	0
Weak erythema	1
Redness, infiltration, papules	2
Redness, edema, papules, blisters	3
Redness, edema, and bullae	4

Table 3 Grading Criteria for Severity of Adverse Reactions

Skin reactions	Extent of reaction	score
no adverse reaction	not have	0
Easy to tolerate, causing minimal discomfortDoes not affect normal life	mild	1
Enough to affect normal life	moderate	2
Obstructing normal life	severe	3

2.3 16S rDNA amplicon sequencing technology

2.3.1 Sequencing section

(1) Extraction of Genomic DNA and PCR Amplification

The genomic DNA of the sample was extracted by CTAB or SDS method, and then the purity and concentration of DNA were detected by agarose gel electrophoresis. An appropriate amount of sample DNA was taken into a centrifuge tube, and the sample was diluted to 1ng/μl with sterile water.

Using diluted genomic DNA as a template, specific primers with barcode were selected based on the sequencing region using Phusion from NewEngland Biolabs ® High-Fidelity PCR Master Mix with GC Buffer,Perform PCR with efficient and high fidelity enzymes to ensure amplification efficiency and accuracy.

Primer corresponding region:
16SV4 region primers (515F and 806R): identification of bacterial diversity;
18SV4 region primers (528F and 706R): identification of eukaryotic microbial diversity;
ITS1 primers (ITS5-1737F and ITS2-2043R): identification of fungal diversity;
In addition, the amplification region also includes: 16SV3-V4/16SV4-V5/16SV5-V7; Archaea 16SV4-V5/Archaea 16SV8; 18SV9 and ITS2 regions.
(2) Mixing and Purification of PCR Products
PCR products were detected by electrophoresis using agarose gel with 2% concentration; The qualified PCR products were purified by magnetic beads, quantified by enzyme label, and mixed in equal amounts according to the concentration of the PCR products. After full mixing, the PCR products were detected by 2% agarose gel electrophoresis. For the target strip, the gel recovery kit provided by Qiagen Company was used to recover the products.
(3) Library construction and machine sequencing
Using TruSeq ® The DNAPCR FreeSamplePreparation Kit library construction kit is used for library construction. The constructed library is quantified using Qubit and Q-PCR. After the library is qualified, NovaSeq6000 is used for machine sequencing.
2.3.2 Bioinformatics Analysis Section
The raw data obtained from sequencing contains a certain proportion of interference data (Dirty Data). In order to make the results of information analysis more accurate and reliable, the raw data is first filtered and concatenated to obtain effective data (CleanData). Then, based on the valid data, noise reduction analysis is performed to generate Amplification Sequence Variants (ASVs). Based on the ASV analysis results, explore the differences in community structure between different samples or groups through PCoA analysis and Beta analysis; Further explore the differences in community structure between grouped samples, and use T-test statistical analysis method to test the significance of differences in species composition and community structure of grouped samples.
2.4 Statistical Methods
SPSS 21.0 statistical software was used for statistical testing, and Shapiro Wilk Test was used for normality testing. If the normality test $p>0.01$, it indicates a normal distribution. Paired sample t-test was performed, and Wilcoxon rank sum test was performed for those who did not follow a normal distribution. $p<0.05$ had statistical significance.

3. Results

3.1Human test

3.1.1 Instrument test results

(1) Test results and statistical analysis of percutaneous water diversion loss

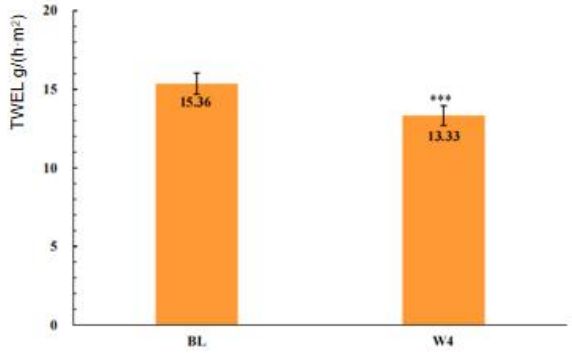

Figure 1. Mean value of percutaneous water diversion loss test results
(BL is the initial mean value before using the product, W4 is the four week mean value after using the product).

As shown in Figure 1, the average value of facial transcutaneous water diversion loss measured by the subjects before using the product was 15.36g/(h•m^2), and the average value of facial transcutaneous water diversion loss measured four weeks after using the product was 13.33g/(h•m^2), a decrease of 13.22% from the initial value. Statistical analysis showed that,p<0.001,Due to significant differences, this product can effectively improve the loss of transdermal water flow in the face of subjects and repair the skin barrier.

(2) Elastic R2 test results and statistical analysis

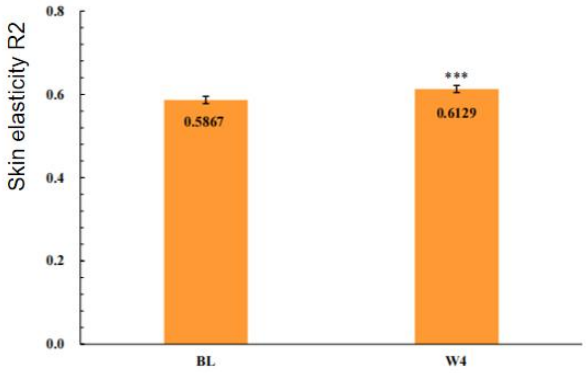

Figure 2. Mean Results of Elasticity R2 Test

(BL is the initial mean value before using the product, W4 is the four week mean value after using the product)

As shown in Figure 2, compared with before using the test product, after using the test product for 4 weeks, the R2 value of cheek skin elasticity significantly increased by 4.47%, and p<0.001. Therefore, this product can significantly improve the facial elasticity level of the subjects.

(3) Smoothness parameter (SEsm) test results and statistical analysis

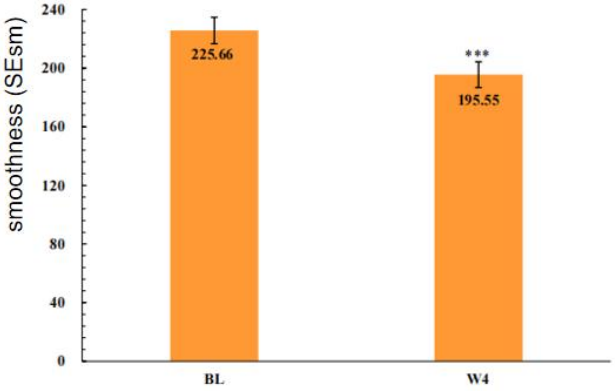

Figure 3. Mean Smoothness SEsm Test Results

(BL is the initial mean value before using the product, W4 is the four week mean value after using the product)

As shown in Figure 3, compared with before using the test product, after using the product for 4 weeks, the subject's cheek smoothness parameter (SEsm) significantly improved by 13.34%, and p<0.001.

(4) Example of Subject Image

Select 2 subjects who showed significant improvement after using the test product for 4 weeks, with subject numbers 011 and 020, as shown in Figure 4 and Figure 5.

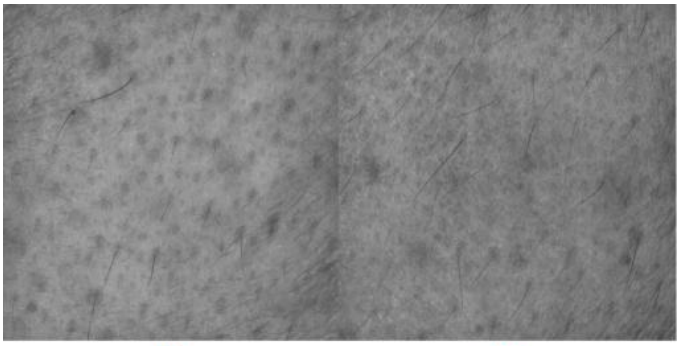

Figure 4. Sample Image of Subject 011- Nourishing (VisioScan VC20 plus Photo)

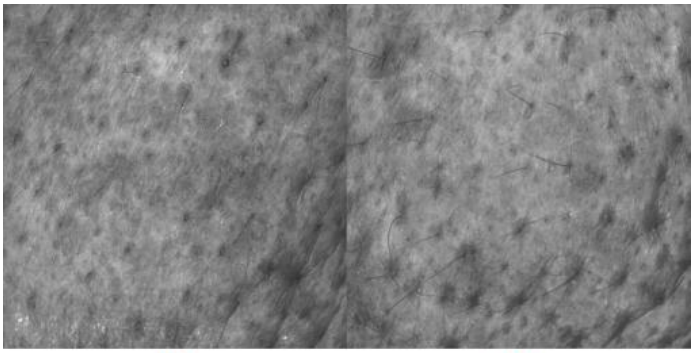

Figure 5. Sample Image of Subject 020- Nourishing (VisioScan VC20 plus Photo)

From Figures 4 and 5, it can be seen that there were significant changes in the skin of the subjects before and after using the product. Figure 4 shows that the subjects' facial skin features were more delicate and their skin tone was more uniform, visible to the naked eye; Figure 5 shows a significant improvement in the dullness of the subject's skin and a more delicate appearance, indicating that this product has a good nourishing effect.

3.1.2 Subject self-assessment results

The overall satisfaction rating of the subjects with this product is high, with overall scores above 84%. The improvement scores for skin smoothness and delicacy are both 90.63%. In terms of skin feel, the product is generally considered to have moderate thinness, easy absorption, and even application. The subjects gave a good evaluation score.

3.1.3 Adverse Reaction Evaluation Results

The adverse reaction results of the subjects are shown in Table 4.

Table 4 Evaluation Results of Adverse Reactions in Subjects

Number of participants	**Local skin adverse reactions**				
	0	1	2	3	4
32	32	0	0	0	0

According to Table 5, none of the subjects experienced adverse skin reactions after using this product, indicating that it is a relatively safe product.

3.2 16S rDNA amplicon sequencing technology

(1) Analysis of inter group differences in Beta diversity index

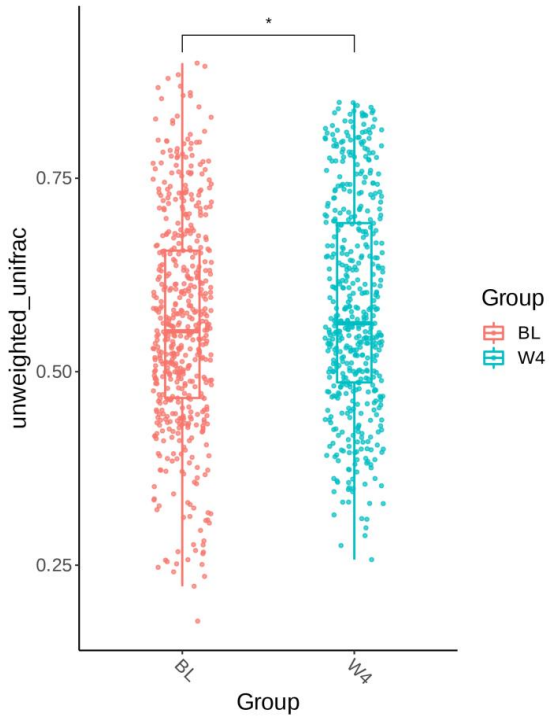

Figure 6. Box plot of unweighted Unifrac Beta diversity based on ASV

The Beta diversity analysis of skin microbiota after using cosmetics is shown in Figure 6. As shown in Figure 6, with the prolonged use of the product, the types of facial microbiota can be significantly clustered and distinguished from the 0-day sample, indicating that the use of this product has a significant impact on the composition of facial skin microbiota.

(2) PCoA analysis

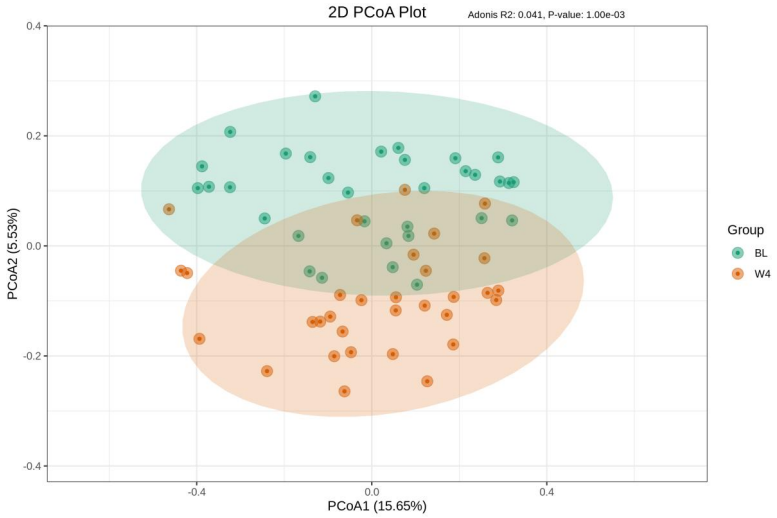

Figure 7. Analysis of Unweighted Unifrac Distance PCoA Based on ASV

According to Figure 7, there were significant differences in the composition of facial microbiota and changes in the structure of facial microbiota types before and after using the sample.

（3） Species Differences

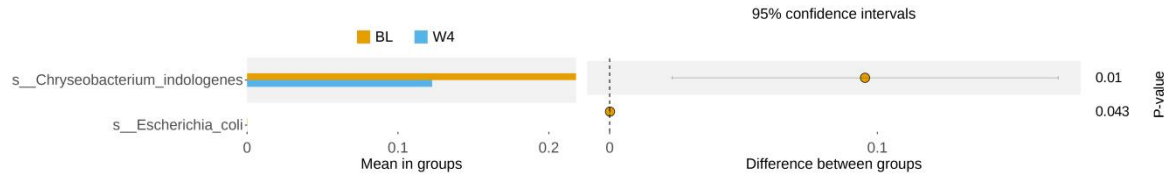

Figure 8. Analysis of Species Differences between T-test Groups Based on ASV

From Figure 8, it can be seen that after using the product, the abundance of harmful bacterium Chryseobacterium decreased, indicating that the product can have an inhibitory effect on this harmful bacterium.

4. Discussion

The active substances derived from biological fermentation technology have many advantages in skincare, such as the ability to break down large molecules (such as collagen and polysaccharides) into small peptides, amino acids, etc. through microbial fermentation, which is more conducive to skin absorption[6]. The fermentation process can reduce the potential irritation of the raw materials and be gentler on the skin[7]. In this study, *YEAST FERMENT EXTRACT* is rich in amino acids, peptides, nucleotides, and vitamins and minerals, which can effectively enhance skin moisture and improve skin brightness. *LACTOBACILLUS/OYMILK FERMENT FILTRATE* contains metabolites of lactic acid bacteria, which can improve the diversity of beneficial bacterial strains on the skin surface and enhance the health of the microecological barrier, At the same time, it can enhance the vitality of epidermal and keratinocytes, promote the physical barrier of the skin[8], It can also inhibit various inflammatory factors and enhance the skin's immune barrier, provide a certain foundation for skin health. *BIFIDA FERMENT LYSATE* is a fermented product derived from Bifidobacterium, which has excellent effects in repairing UV damage and photoaging, helping the skin resist oxidation and photodamage, and delaying aging. The above three active substances can synergistically promote each other, and have comprehensive effects on skin barrier and micro ecological health, whitening, brightening, anti-aging, etc[9]. They have good nourishing and promoting skin health effects. And the product is mild and non irritating, with good application prospects in the fields of skin microecology and green natural skincare.

5. Conclusion

This essence liquid, which is mainly composed of three kinds of fermentation materials, is in line with the current situation of the development of microbiological technology and fermentation technology in the cosmetics field. After four weeks of use, this product can significantly reduce the loss of skin water diversion of the subjects by 13.22%, significantly improve the cheek elasticity R2 of the subjects by 4.47%, significantly improve the cheek smoothness parameter SEsm by 13.34%, and the subjective evaluation scores of the subjects are all above 84%, and there is no adverse reaction in the test of 32 people. The overall analysis shows that this product is a safe product with good nourishing effect. After 16S rDNA sequencing analysis[10], it was found that the facial microbiota of the subjects had undergone changes in deconstruction before using the product compared to after using the product for 4 weeks, and the composition of the microbiota was healthier. Among them, the abundance of harmful bacteria such as indole producing golden yellow bacillus decreased, achieving the effect of regulating the balance of facial microbiota in the subjects[11].

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