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ORIGINAL ARTICLE

The pivotal role of *Bifida Ferment Lysate* on reinforcing the skin barrier function and maintaining homeostasis of skin defenses in vitro

Rui Wang ME[1,2] | Shiyu Yan ME[1,2] | Xue Ma ME[1,2] | Jinfeng Zhao ME[1,2] | Yuqing Han ME[1,2] | Yao Pan PhD[1,2] | Hua Zhao PhD[1,2]

[1]Department of Cosmetics, College of Chemistry and Materials Engineering, Beijing Technology and Business University, Beijing, China

[2]Beijing Key Laboratory of Plant Research and Development, Beijing, China

Correspondence
Yao Pan, Department of Cosmetics, College of Chemistry and Materials Engineering, Beijing Technology and Business University, 11 Fu Cheng Road, Hai Dian District, Beijing 10048, China.
Email: panyao@btbu.edu.cn

Abstract

Background: The semiactive or inactive probiotics or their extracts used in dermatology have interesting properties to ameliorate signs of irritated skin and enhance the skin barrier. *Bifidobacterium*, as the most common probiotics, which has been found to be effective in reducing acne and improving the skin barrier function of atopic dermatitis. *Bifida Ferment Lysate* (BFL) can be obtained from *Bifidobacterium* by fermentation and extraction.

Purpose: In this study, we investigated the effect of a topically used BFL on the skin using in vitro evaluation methods.

Results: The results showed that upregulation of skin physical barrier gene (FLG, LOR, IVL, TGM1, and AQP3) and antimicrobial peptide gene (CAMP and hBD-2) in HaCaT cells by BFL might be responsible for skin barrier resistance. In addition, BFL had strong antioxidant properties representing a dose-dependent increasing of the scavenging capacity of DPPH, ABTS, hydroxyl, and superoxide radicals. BFL treatment also fundamentally inhibited the intracellular ROS and MDA production and improved the activities of antioxidant enzymes (CAT and GSH-Px) in H_2O_2-stimulated HaCaT cells. As a good immunomodulatory factor, BFL efficiently decreased the secretion of IL-8 and TNF-α cytokines, and COX-2 mRNA expression in LPS-induced THP-1 macrophages.

Conclusion: BFL can strengthen the skin barrier function and stimulate skin barrier resistance, to reinforce the skin against oxidative stress and inflammatory stimuli.

KEYWORDS
antioxidation, *Bifida Ferment Lysate*, immunomodulation, probiotics, skin barrier resistance

Rui Wang and Shiyu Yan contributed equally to this work and should be considered co-first authors.

1 | INTRODUCTION

The skin covers the whole body to form an effective barrier against the external environment, which plays a crucial role in transcutaneous water loss counterbalance, temperature maintenance, and immune surveillance.[1,2] Strugar et al. described the skin barrier as four main components: the immune, physical, chemical, microbiome layer.[3] The immune layer senses danger signals through the molecular patterns related to pathogens and damage, and resident immune cells work to eliminate invasion.[3,4] The physical layer is composed of corneum, whose main functions include preventing water loss and protecting against irritants from penetrating,[5] and it mainly refers to the sebum membrane, keratin, lipids, brick wall structure, dermal mucopolysaccharides, and mucopolysaccharides.[6] The chemical layer is distributed throughout the physical layer, including natural moisturizing factors, defensive chemicals, and ingredients which maintain the acidic skin pH.[7] The microbial layer is composed of living organisms, which will form a stable environment related to protective immune response.[8] As the skin barrier is highly organized, the normal metabolism of each part is essential for its protection. Damage to the skin barrier will boost the penetration of external antigens and stimuli that may provoke skin inflammation, such as atopic dermatitis, acne, chronic actinic dermatitis, and psoriasis.[9–12]

The skin microbiota contributes significantly in the suppression of pathogenic species and regulation of skin proteins, free fatty acids, and lipid-rich substances,[13,14] which is an important part of the skin barrier. Studies have shown that probiotics are healthy and potential living microorganisms, which impact on skin inflammation and skin homeostasis by deploying probiotics.[15,16] The local utilization of inactivated probiotics components has proved to be persuasive in strengthening the skin barrier of acne, atopic dermatitis and other skin diseases.[17] In addition, the fermented extracts of probiotics have also been proved to be valuable on the skin barrier. In a study to explore the effect of *Lactobacillus rhamnosus* on the reconstruction of skin barrier function in human epidermal model, it was found that *Lactobacillus rhamnosus lysate* reduced the cytotoxicity of a skin irritant (sodium lauryl sulfate), and enhanced the protection of skin barrier.[18] The application of *Vitreoscilla filiformis* fermentation extract on the skin not only strengthens the skin barrier, but also enhances the immune defense.[19] As one of the most common microorganisms in probiotics, *Bifidobacterium* is also an important source of probiotic strains.[16,20] Importantly, recent interesting data indicate that *Bifidobacterium*-based topical formulations have shown good results in reducing acne and improving the skin barrier function of atopic dermatitis.[21,22]

Bifida Ferment Lysate (BFL) is the fermentation extract, metabolites, cytoplasmic fragments, cell wall components, and polysaccharide complex obtained by culture, inactivation, and decomposition of *Bifidobacterium*. BFL can produce beneficial skin care small molecule substances, such as amino acids, vitamin B group, minerals. In a recent clinical trial, it was found that a topical product containing gel-based BFL was safe and effective in the treatment of mild or moderate acne.[23] In addition, an in vitro study showed that a mixed formulation containing BFL could protect skin from free radicals.[24] In view of the potential results revealed by existing tests, none of the effects of BFL on skin health has been comprehensively and widely discussed, it is necessary to carry out further research. In this study, a BFL isolated from *Bifidobacterium* was used, and its beneficial impact on skin was studied in vitro. The skin barrier enhancement effect of BFL was studied on HaCaT cells by the expression of both keratinocyte differentiation markers (physical barrier) and antimicrobial peptides (AMPs, biochemical barrier). The oxidative stress resistance effect of BFL were detected by free radical scavenging assays and an H_2O_2-induced oxidative damage HaCaT cell model. In addition, the immunomodulatory effect of BFL was evaluated in lipopolysaccharide (LPS)-stimulated THP-1 monocyte-derived macrophages.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Ferrous sulfate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), salicylic acid, hydrogen peroxide, lipopolysaccharide (LPS), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich. PBS was purchased from Hyclone. DPPH were purchased from Tokyo Chemical Industry. Tris and pyrogallol were purchased from Biotopped. BFL was purchased from CLR Berlin.

2.2 | Cell culture

Human HaCaT cells and THP-1 cells were purchased from the National Infrastructure of Cell Line Resource, and cultured in MEM cell medium and RPMI-1640 cell medium (National Infrastructure of Cell Line Resource), respectively. The culture medium was supplemented with 10% FBS (Gibco) and 1% penicillin–streptomycin (Gibco). The macrophage-like state was induced by treating THP-1 monocytes (1×10^6 cells/well) for 24 h with 100 ng/mL PMA in 24-well cell culture plates.

2.3 | Free radical scavenging activity assay

2.3.1 | DPPH scavenging activity assay

The DPPH method was used to detect the free radical scavenging ability of BFL. Briefly, the BFL solution with a concentration of 0.1%–30% was added to DPPH solution of 2.0×10^{-4} mol/L in a 96-well plate and incubated for 30 min at room temperature without light, and the absorbance was measured at 517 nm.

2.3.2 | ABTS scavenging activity assay

The ABTS radical scavenging activity assay was carried out based on the total antioxidant capacity assay kit with the ABTS method instructions (Beyotime). BFL was mixed with ABTS working solution at different concentrations and incubated for 2–6 min at room temperature, and the absorbance was measured at 734 nm.

2.3.3 | Hydroxyl radical scavenging activity assay

The hydroxyl radical scavenging ability of BFL was determined by the salicylic acid method, and 6 mL of BFL samples of different concentrations were added into the reaction system of the same volume (9 mmol/L ferrous sulfate solution, 0.4 mL of 0.3% H_2O_2, and 0.2 mL of 9 mmol/L salicylic acid-ethanol). Finally, the reaction was carried out at 37°C for 15 min. The absorbance of the mixture was measured at 510 nm.

2.3.4 | Superoxide radical scavenging activity assay

Pyrogallol is oxidized under alkaline conditions to produce colored intermediates and superoxide radicals ($O_2\cdot^-$). 100 mmol/L Tris–HCl buffer solution (2.8 mL, pH 8.2) and BFL (0.1 mL) was mixed at different concentrations in a 10-mL test tube and kept warm in 37°C water bath for 20 min. 3 mmol/L catechol (0.1 mL) was added and preheated at 37°C, and then the absorbance was measured at 325 nm.

2.4 | Cell viability assay

The MTT method was used to evaluate cytotoxicity. HaCaT cells were seeded in 96-well plates for 24 h, with a density of 1×10^4 cells/well. After 24 h removing the medium, the cells were given treatments for 24 h with BFL at a set concentration of 0.15625%–20%. For THP-1 macrophages, the cells were cultured in 96-well plates at 1×10^5 cells/well, and were treated with BFL (0.15625%–20%) for 24 h. 10 μL MTT (5 mg/mL) was added to each well and incubated at 37°C for 4 h. After incubation, 100 μL lysis solution containing 5% isobutanol, 10% SDS, and 12 mM HCl in H_2O were put into each well to solubilize the formazan crystal. The absorbance was measured at 570 nm using a Tecan Infinite M200 Pro multi-mode microplate reader (Tecan Trading AG).

2.5 | Intracellular catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), malondialdehyde (MDA), and reactive oxygen species (ROS) determination

HaCaT cells were cultured at 3×10^5 cells/well in a six-well plate at 37°C for 24 h. After pretreated with 0.003% H_2O_2 for 6 h, the cells were cultured for 18 h by adding different doses of BFL. After trypsin digestion, the cells were centrifuged at 1000 r/min for 5 min, and the contents of CAT, GSH-Px, SOD, and MDA were detected according to the instructions of commercial CAT, GSH-Px, SOD, and MDA detection kits (Beyotime). The OD values in each group were measured at the corresponding wavelength. For ROS determination, added DCFH-DA (1 mL) diluted with serum-free culture medium with a volume ratio of 1:1000 to the cells. Incubated in a 37°C cell incubator for 20 min. The cells were washed three times with serum-free cell culture medium to fully remove DCFH-DA that had not entered the cells. Flow cytometry (BD Accuri C6, BD Biosciences) was used to detect intracellular ROS content by its fluorescence intensity.

2.6 | Secretion of inflammatory cytokines

The THP-1 macrophages were stimulated with 1 μg/mL LPS for 18 h. After stimulation, 1%, 3%, and 5% BFL were added to the sample wells, and culture medium was added to the blank wells and LPS control wells to further incubate for 6 h.

The cytokine concentrations of IL-8 and TNF-α in the THP-1 macrophages supernatants were measured with human IL-8 and TNF-α ELISA kits according to the protocols provided by the manufacturer (Invitrogen).

2.7 | RT-PCR

Total RNA was obtained from HaCaT cells and THP-1 macrophages used a TIANGEN RNA Kit (TIANGEN BIOTECH CO., LTD). Reversely transcribed RNA (1 μg) into cDNA using ReverTra Ace qPCR RT Kit (TOYOBO) according to the kit instructions. RT-PCR was performed on a CFX96 Real-time System (LightCycler®480 II, Roche) using SYBR Green Realtime PCR Master Mix (TOYOBO). The primers are shown in Table 1. The conditions for amplification were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 55°C for 10 s, and 72°C for 15 s. Real-time quantification Ct values were gathered and the target gene expression was gave the go-ahead to GAPDH using the $2^{-\Delta\Delta Ct}$ method.

2.8 | Western blot analysis

HaCaT cells were treated with BFL (1%, 3%, and 5%) for 24 h. Cells were lysed with cell lysis buffer for western and immunoprecipitation (IP) (Beyotime), and cell debris was removed via centrifugation. The concentrations of protein from the supernatant were quantitated using the BCA Protein Assay Kit (Beyotime). After quantitation, equal amounts of protein were loaded on a sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE; Beyotime) and transferred into a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with a QuickBlock™ Western Occluder (Beyotime) and incubated at 1:1000 primary antibody overnight at 4°C. The membranes

TABLE 1 Primer sequences used for RT-PCR.

Primer	Sequence 5′–3′
FLG- Forward	GTTACAATTCCAATCCTGTTGTTTTC
FLG- Reverse	CGTTGCATAATACCTTGGATGATC
LOR- Forward	GGCTGCATCTAGTTCTGCTGTTTA
LOR- Reverse	CAAATTTATTGACTGAGGCACTGG
IVL- Forward	AGCCTTACTGTGAGTCTGGTTGA
IVL- Reverse	GGGTATTGACTGGAGGAGGAACA
AQP3- Forward	GCTGTCACTCTGGGCATCCTC
AQP3- Reverse	GCGTCTGTGCCAGGGTGTAG
TGM1- Forward	GATCGCATCACCCTTGAGTTAC
TGM1- Reverse	GCAGGTTCAGATTCTGCCC
CAMP- Forward	GCTAACCTCTACCGCCTCCT
CAMP- Reverse	GGTCACTGTCCCCATACACC
hBD-1- Forward	TCTGCTGTTTACTCTCTGCTTAC
hBD-1- Reverse	GGCAGGCAGAATAGAGACATT
hBD-2- Forward	GGTGTTTTTGGTGGTATAGGC
hBD-2- Reverse	AGGGCAAAAGACTGGATGACA
hBD-3- Forward	CCAGGTCATGGAGGAATCATAAA
hBD-3- Reverse	CGATCTGTTCCTCCTTTGGAAG
COX-2- Forward	CCCACCCATGTCAAAACCGA
COX-2- Reverse	CCGGGTACAATCGCACTTATACT
GAPDH- Forward	CTTTGTCAAGCTCATTTCCTGG
GAPDH- Reverse	TCTTCCTCTTGTGCTCTTGC

were treated with a horseradish peroxidase-conjugated (HRP) secondary antibody for 2 h at a diluted concentration of 1:5000 and were visualized using a Tanon-5200 Multi System (Tanon). The expression level of each protein was analyzed using ImageJ software and normalized to β-actin. The following primary antibodies were used for western immunoblotting: filaggrin (FLG) and β-actin.

2.9 | Statistical analysis

Statistical analysis was executed by SPSS software version 22. Student's *t*-test was used to compare the difference between two groups, while one-way ANOVA pursued by Dunnett's post hoc was used for comparison of multi-dose BFL treatment groups with the control group. All experiments were carried out in triplicate, and the results are expressed by mean ± SD of three individual experiments. When $p < 0.05$, differences were considered as statistically significant.

3 | RESULTS

3.1 | Effect of BFL on cell viability in HaCaT and THP-1 macrophages

As shown in Figure S1A, the cell viabilities of HaCaT cells and THP-1 macrophages were not impacted by BFL (0.15625%–10%). To further study the health benefits of the BFL, low, medium, and high doses of 1%, 3%, and 5% BFL, respectively, were selected for the following experiments (Although the cell viability test showed that 10% of the BFL was safe to both cell models, we plan to apply 5% of this ingredient in the product as the highest dose. Therefore, we used 5% as the high dose in this in vitro efficacy study).

3.2 | Effect of BFL on barrier maintenance-associated molecules in HaCaT cells

Filaggrin, loricrin (LOR), involucrin (IVL), transglutaminase 1 (TGM1), and aquaporin 3 (AQP3) genes are important to maintain skin barrier function. The mRNA expression levels of FLG, LOR, IVL, and AQP3 genes expression were increased in a dose-dependent manner following BFL treatment at the various concentrations (Figure 1A). The expression of FLG protein in HaCaT cells was determined as well. BFL treatment also dose-dependently augmented FLG protein levels (Figure 1B). Cathelicidin (CAMP) and β-defensin (hBD-1, hBD-2, hBD-3) are the main AMPs in the epidermis that has the function of antibacterial and skin barrier maintenance. The results showed that the mRNA expressions of CAMP and hBD-2 in HaCaT cells were significantly increased after treating with different concentrations of BFL, while the enhancement of hBD-1 and hBD-3 mRNA levels was not significant (Figure 1C). These results suggested that BFL might promote the skin barrier maintenance function by up-regulating the expression of skin physical and antimicrobial barrier-related molecules in HaCaT cells.

3.3 | Free radical scavenging activity of the BFL

The scavenging ability of DPPH, ABTS, hydroxyl radicals, and superoxide radicals raised in a dose-dependent manner after exposure to six concentrations of BFL (0.1%–30%). The results showed that the scavenging rates of the 30% BFL to these four radicals were 34.42%, 54.34%, 63.75%, and 13.66%, respectively (Figure 2A–D). It indicated that the scavenging ability of BFL for these four free radicals was ranked as hydroxyl radical > ABTS > DPPH > superoxide radical.

3.4 | Effect of BFL on antioxidative capacity in HaCaT cells

After treating with 0.003% H_2O_2, the antioxidase activities of CAT, SOD, and GSH-Px were significantly decreased while the intracellular ROS and MDA contents were increased in HaCaT cells, indicating that an oxidative damage cell model was successfully established. Cells exposed to BFL exhibited significantly ($p < 0.05$) raised CAT and GSH-Px activities and inhibitory effects on H_2O_2-induced ROS and MDA production in a dose-dependent manner (Figure 3B–F). These findings revealed that BFL protected HaCaT cells by suppressing the ROS and MDA produced in response to H_2O_2 exposure, thus the antioxidant capacity of cells was improved.

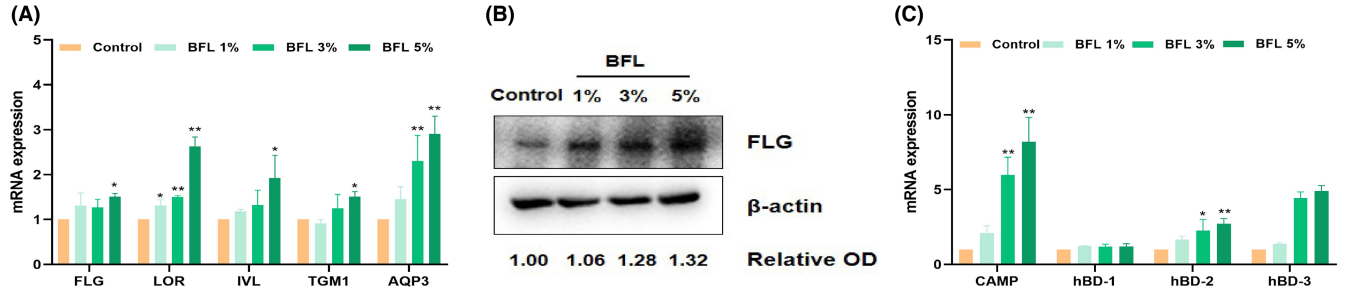
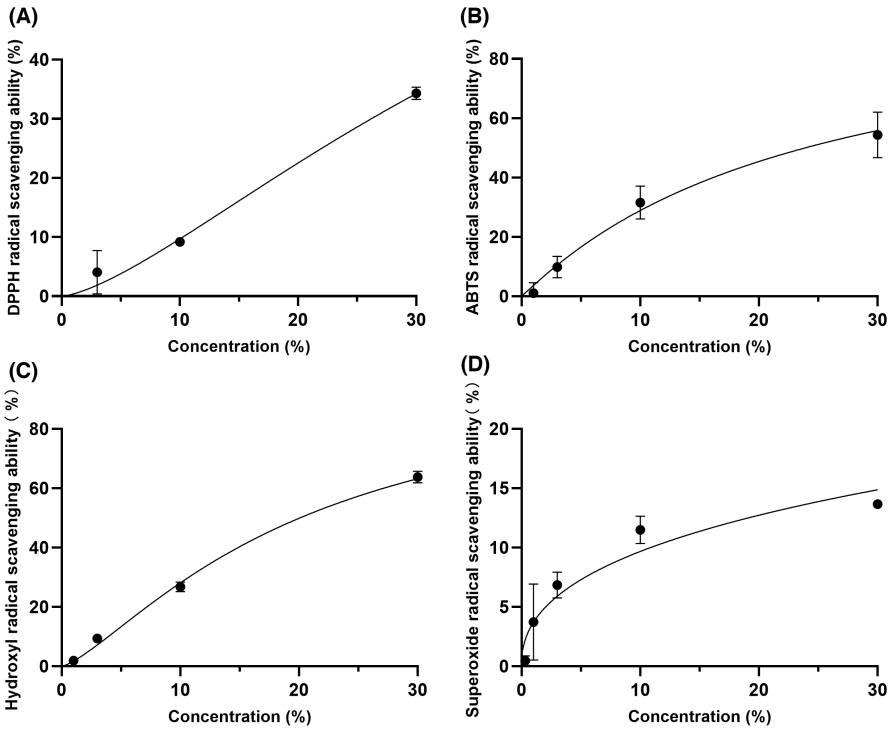


FIGURE 1 Effect of BFL on the expression of physical barrier-associated molecules and antimicrobial peptide genes in HaCaT cells by RT-PCR. HaCaT cells were treated with 1%, 3%, and 5% concentrations of BFL for 24 h. (A) Skin physical barrier genes-FLG, LOR, IVL, AQP3, and TGM1 mRNA expression levels. (B) Skin barrier molecule FLG protein expression level. (C) Skin antimicrobial peptide genes- CAMP, hBD-1, hBD-2, and hBD-3 mRNA expression levels. Data are shown as mean ± standard deviation ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

FIGURE 2 Scavenging activity of BFL (0.1%, 0.3%, 1%, 3%, 10%, 30%) on (A) DPPH, (B) ABTS, (C) hydroxyl radical, and (D) superoxide radical by free radical scavenging experiments. Values are represented as a percentage of the control. Data are shown as mean ± standard deviation ($n = 3$).



3.5 | Effect of BFL on immunomodulatory function of THP-1 macrophages

As shown in Figure 4A,B, the cytokine secretion levels of IL-8 and TNF-α, and the mRNA expression level of cyclooxygenase-2 (COX-2) in THP-1 macrophages were dramatically increased after LPS stimulation ($p < 0.01$), which suggested that the inflammatory cell model was successfully established. Compared with the LPS stimulation group, BFL significantly inhibited IL-8 and TNF-α production in THP-1 macrophages ($p < 0.01$). In accordance with the cytokine secretion, COX-2 mRNA expression in LPS-stimulated THP-1 macrophages were significantly decreased after treatment with different concentrations of BFL.

4 | DISCUSSION

The skin is the largest organ of the human being, which forms an effective barrier between the "inside" and "outside" of the body.[1] Therefore, maintaining skin barrier function constitutes a major therapy for various dermatological diseases.[25] Probiotics are live microorganisms used for various health benefits on the host.[26] In recent years, clinical studies have reported that probiotics play an important role in improving the skin barrier.[27] In this study, we evaluated the beneficial effects of BFL, a probiotic, on skin health by investigating barrier enhancement and resistance to barrier damage stimuli.

The skin barrier is composed of physical, chemical/biochemical, and immune barriers,[1] which is closely related to the transcription and translation of skin barrier genes. The production of epidermal terminal differentiation molecules (TGM1, FLG, and IVL) are pivotal for maintain skin barrier function.[28] LOR plays an essential role in the skin barrier function and is a major component of the cornified envelope, which binds to filaggrin to form a cornified envelope.[29] AQP3 is the most important channel protein in human skin. Epidermal moisture content and water loss have close association with the expression of AQP3.[30] AMPs in the skin directly stimulate

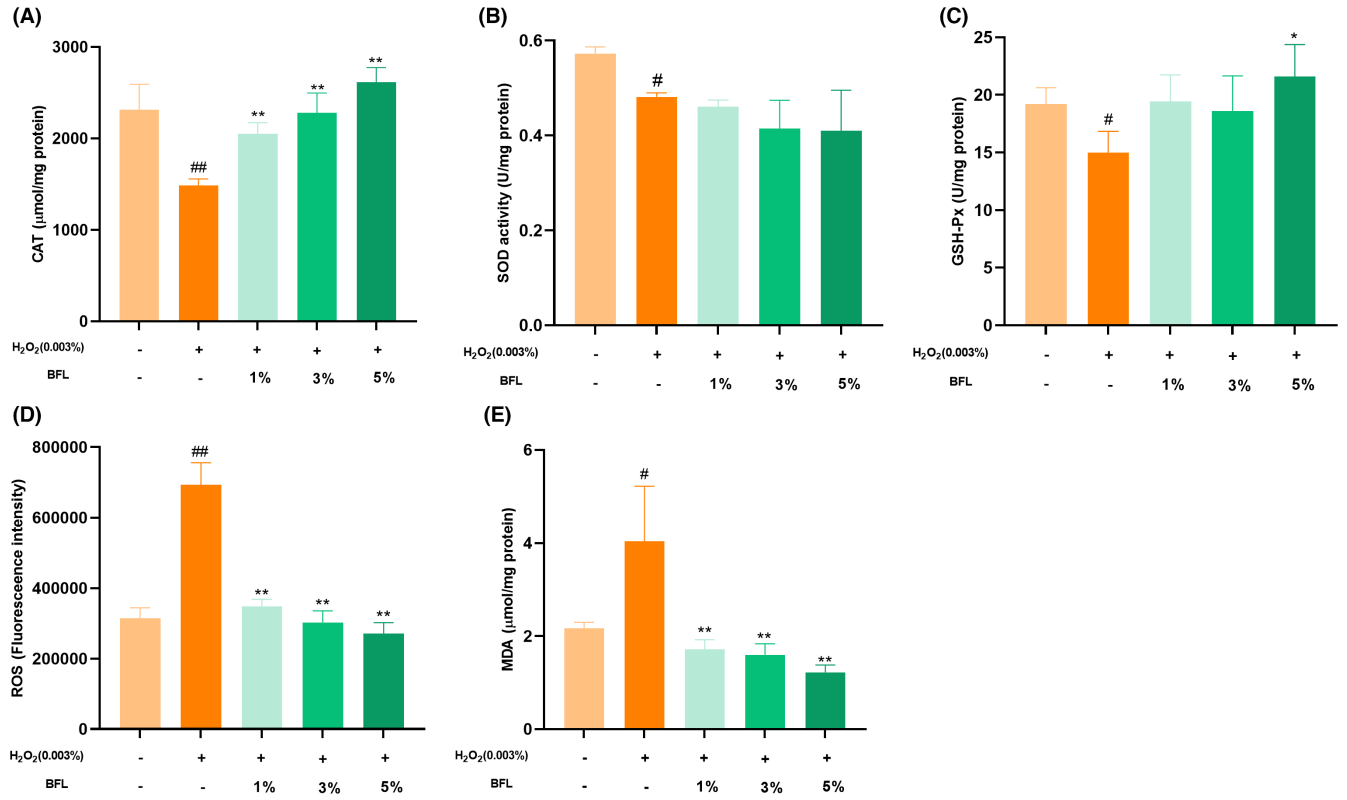


FIGURE 3 Antioxidant capacity of BFL on HaCaT cells. Effect of BFL on increasing the antioxidant enzymes (A) CAT, (B) SOD, and (C) GSH-Px, and decreasing (D) ROS content and (E) MDA in H_2O_2-stimulated HaCaT cells. Data are shown as mean ± SD ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences compared with the H_2O_2 stimulation group; $\#p < 0.05$ and $\#\#p < 0.01$ indicate significant differences compared with the control group.

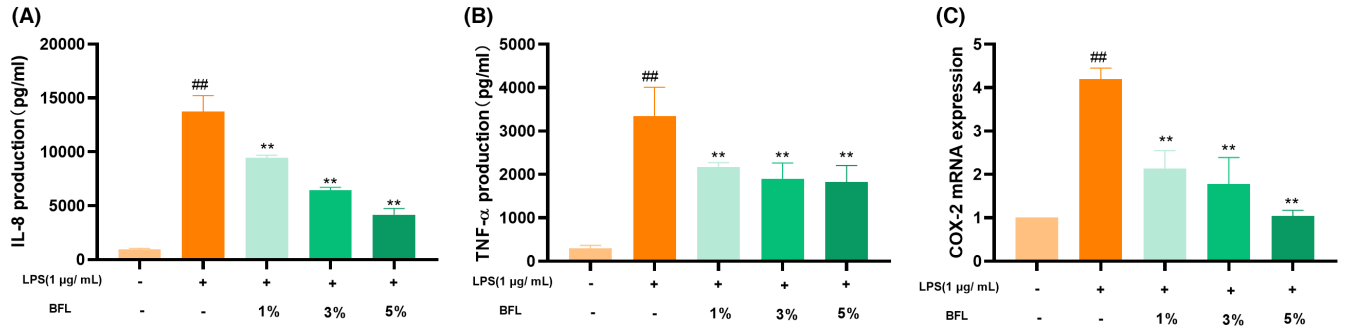


FIGURE 4 Anti-inflammatory effect of BFL on THP-1 macrophages. The production of (A) IL-8 and (B) TNF-α was quantified by ELISA, and (C) COX-2 mRNA expression was detected by RT-PCR. Data are shown as mean ± standard deviation ($n = 3$). $^{**}p < 0.01$ indicates significant differences compared with the LPS stimulation group; $\#\#p < 0.01$ indicates significant differences compared with the control group.

pathogens through antibacterial effects or indirectly through inflammatory pathways, providing the first-line immune defense against pathogens.[31,32] Attila et al. found that *Bifidobacterium longum* extract effectively improved the expression of barrier gene TGM1 and antimicrobial peptide genes in normal human epidermal keratinocytes.[33] In addition, *Vitreoscilla filiformis* extract showed to strengthen the skin barrier by increasing the expression of FLG, IVL, and antimicrobial peptide β-defensin-4A in normal human epidermal keratinocytes.[19] In our study, BFL enhanced skin physical and antimicrobial barrier-associated genes in HaCaT cells, as shown by increasing FLG, LOR, IVL, AQP3, TGM1, CAMP, and hBD-2 mRNA expression levels (Figure 1A,C). Moreover, a dose-dependent rise in FLG protein levels was also observed after BFL treatment (Figure 1B). We considered that BFL promoted the skin barrier by inducing the expression of keratinocyte differentiation molecules and antimicrobial peptide genes.

Since skin defends the body from the external harmful environments, it would be the most influenced organ from oxidative stress as a result of exogenous ROS stimuli.[34,35] A large variety of environmental factors such as solar ultraviolet (UV) light, visible

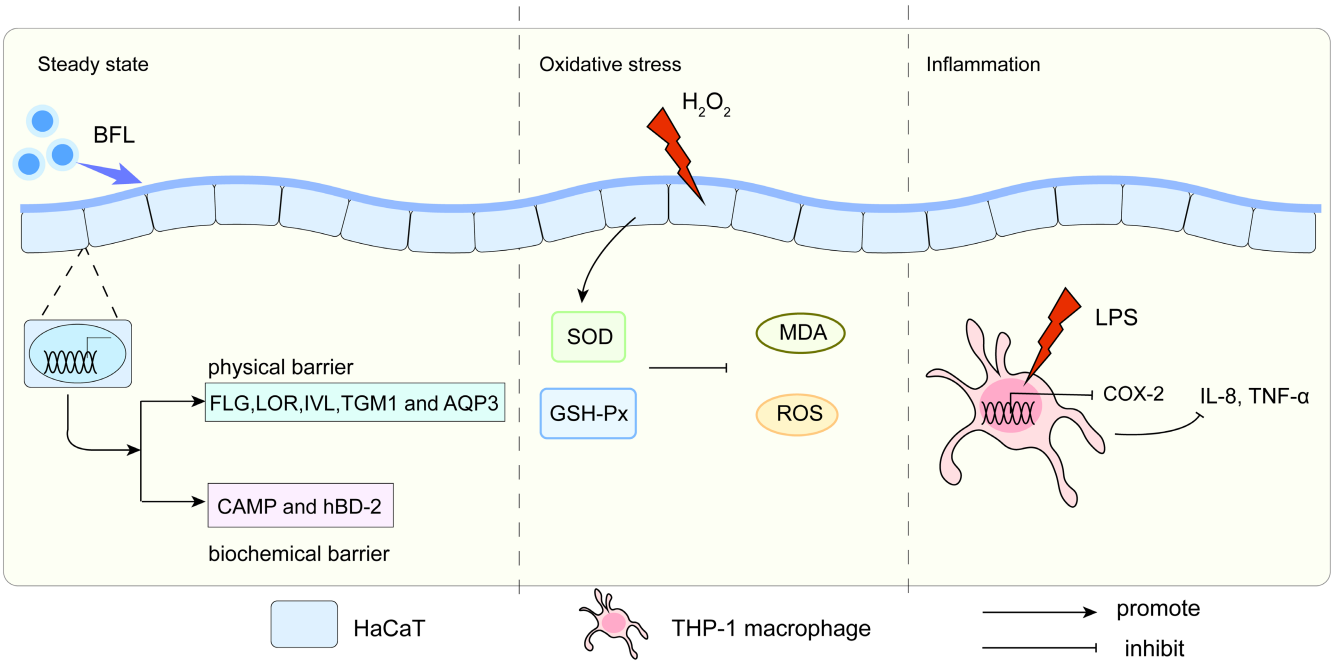


FIGURE 5 Schematic presentation of the effects of BFL on strengthening the skin barrier function and defensing oxidative stress and inflammation.

light, environmental pollution, including ozone and particulate matter, increase the production of ROS in the skin.[36,37] Oxidative stress and its resulting oxidative damages can exacerbate skin barrier disruption. Furthermore, skin barrier deficiency and inflammatory response each is highly relevant to the other.[38,39] In the process of skin inflammation, the formation of pro-inflammatory cytokines will interfere with the permeability of the barrier, thus affecting the normal function of the skin.[40] In this study, we found that BFL efficiently scavenged free radicals in a dose-dependent manner (Figure 2). At the cellular level, BFL treatment radically boosted antioxidant enzymes (CAT and GSH-Px) activities (Figure 3A,C), reduced intracellular ROS contents (Figure 3D), and inhibited the release of MDA (Figure 3E) in H_2O_2-induced HaCaT cells. According to the prominent scavenging activities of BFL on hydroxyl radicals in vitro, we speculated that BFL might attenuate H_2O_2-stimlated cell oxidative damage by scavenging the content of hydroxyl radicals, as well as promoting the antioxidant capacity of HaCaT cells. The cell-free culture supernatant produced by *Bifidobacterium longum* subsp. exhibited strong scavenging activity on DPPH and hydroxyl radicals, which was consistent with our study.[41] This manifested that BFL exhibited a strong radical scavenging and anti-oxidant effect. A study by Kang et al. showed that *Lactobacillus buchneri* extract significantly increased the activity of CAT and SOD in UVB-damaged fibroblasts and HaCaT keratinocytes.[42] It is worth noting that we found BFL could suppressed the content of inflammatory cytokines and mediators, including IL-8, TNF-α, and COX-2 in LPS-induced THP-1 macrophages (Figure 4A–C). Previous research displayed that *Limosilactobacillus reuteri* PTCC 1655 cell-free culture supernatant significantly decreased THP-1 macrophages cytokines such as TNF-α, IL-1β, and COX-2.[43] Combined with these results, we believed that BFL could ameliorate the balance of the skin barrier by effectively scavenging free radicals, resisting to oxidative stress and boosting cellular immune function, thus restoring the skin barrier function.

Probiotics preparations present tremendous potential to promote skin health, as well as prevent and treat skin diseases. Our study provides emerging insight into the beneficial effects of BFL on increasing epidermal terminal differentiation molecules, antimicrobial peptide synthesis, free radical scavenging capacity, and protecting cellular antioxidation and immune defense depicted in Figure 5. Therefore, BFL can strengthen the skin barrier function and stimulate skin defenses, to reinforce the skin against oxidative stress and inflammatory stimuli.

AUTHOR CONTRIBUTIONS

Rui Wang and Shiyu Yan substantially contributed to the acquisition of the data, and to the analysis and interpretation of the data. Rui Wang and Shiyu Yan were involved in drafting the manuscript or revising it critically for important intellectual content. Xue Ma, Jinfeng Zhao, and Yuqing Han substantially contributed to the acquisition and analysis of the data, Yao Pan substantially contributed to the conception and design. All the authors were fully involved in drafting the final version of the article.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest in this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICAL APPROVAL

The author of the paper guarantees that the paper is an original work and that it does not involve any academic misconduct such as breach of confidentiality or multiple submission. The author of the paper guarantees that the attribution of the paper will not be disputed. None of the authors have a conflict of interest to disclose.

ORCID

Xue Ma https://orcid.org/0000-0002-4937-9934
Yao Pan https://orcid.org/0000-0002-8092-4865

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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