Original paper

***Lactobacillus Plantarum* CGMCC8198-Fermented Milk Inhibits Melanogenesis in B16F10 Melanoma Cells**

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Abnormal accumulation of melanin causes unaesthetic hyperpigmentation. Many studies suggest that yogurt have some effects of antioxidant and anti-aging. In this study, the antioxidant and melanogenesis-inhibitory activity of fermented milk supernatant (FMS) prepared by *Lactobacillus plantarum* CGMCC8198, a novel probiotics strain, were detected. The results in both ABTS and DPPH assay showed that FMS exhibited markedly antioxidant effect. Melanin production was significantly reduced by FMS, while the survival of cells was not affected. Furthermore, FMS reduced the activity of cellular tyrosinase but had no effect on tyrosinase itself, and the results of RT-PCR, Western blot and immunocytochemistry demonstrated that FMS could inhibit the transcription and expression of tyrosinase, TRP-1, TRP-2 and MITF, and these effects were mainly depended on heated-resistant macromolecule substances. Our results suggested that *Lactobacillus plantarum* CGMCC8198-Fermented Milk could be a good candidate for antioxidant and treating hyperpigmentation disorders and could also be used as a cosmetic whitening agent.

Keywords: lactobacillus-fermented milk, antioxidant, melanogenesis, tyrosinase, MITF

Introduction

Melanin plays an important role in the protection of skin against UV, but abnormal accumulation of this pigment causes unaesthetic hyperpigmentation (Beani, 2014). Since the rate-limiting steps in melanogenesis is catalyzed by the tyrosinase family, which includes tyrosinase (TYR), tyrosinase related protein 1 (TRP-1), tyrosinase related protein 2 (TRP-2) (Brenner & Hearing, 2008), and microphthalmia-associated transcription factor (MITF), the key transcription factor of tyrosinase family, are also required for melanin synthesis, the inhibitors of tyrosinase family and MITF are regarded as blockers of melanogenesis and skin hyperpigmentation. However, many existing chemical inhibitors of

tyrosinase have restricted uses because of side-effects or low stability. For example, kojic acid is cytotoxic and associated with side-effects such as dermatitis, while ascorbic acid has very low stability (Draelos, 2007; Koo *et al.*, 2010).

In addition, skin hyperpigmentation and many other skin disorders are always caused by oxidants such as reactive oxygen species (ROS) induced by ultraviolet light (UV) from sunshine, smoke, pollutants, etc (Solano *et al.*, 2006), and melanogenesis also produces the reactive oxidants including hydrogen peroxide (H₂O₂) and ROS, which create oxidative stress in melanocytes (Godic *et al.*, 2014; Kim, M. *et al.*, 2015). Therefore, certain ROS scavengers and inhibitors can inhibit melanogenesis and skin

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pigmentation disorders. For example, reduced glutathione (GSH) and ascorbic derivatives are applied to treat various skin problems such as depigmentation of hyperpigmented spots (Panich *et al.*, 2011; Quevedo *et al.*, 2000). Therefore, discovering drugs with antioxidant activities as well as anti-tyrosinase effects seems necessary for whitening agents. In recent years, although plenty of synthetic drugs are proposed for the treatment of skin diseases, the observed side effects have stopped their progression, development of effective anti-melanogenic agents with antioxidative capacity is still a promising strategy to prevent or improve skin against damage due to UV radiation.

Probiotics is a kind of active microbes that beneficial to the host, and its engraftment in human intestine, reproductive or other physiological system, can produce accurate health benefits to improve the host microecological balance. The most common probiotics are the lactic acid bacteria, which can prevent overgrowth of pathogenic bacteria in the intestine and are also involved in immune regulation, lowering serum cholesterol and many other health protection events (Parvez *et al.*, 2006). Besides, previous studies have demonstrated that lactic acid bacteria also have beneficial effects on skin, such as therapeutic effects on atopic dermatitis, allergies, and photo-aging (Kim, H. R. *et al.*, 2015). Furthermore, when the milk is fermented by lactic acid bacteria, the nutrients are improved and some material with physiological activity, such as organic acid, antibiotics, exopolysaccharides (EPS), active enzyme, etc, is produced. These substances can adjust the health of body and even prevent and cure some diseases (Broadbent *et al.*, 2003). In addition, many studies have suggested that yogurt also have some effects of antioxidant and anti-aging (Virtanen *et al.*, 2007).

Lactobacillus plantarum CGMCC8198 is a novel probiotics strain isolated by our laboratory. Our previous study has proved that it could strongly resist the inhibitory effects of bile salts and reduce blood lipids in mice with hyperlipemia (Gu *et al.*, 2014). However, other health-care functions of *L. plantarum* CGMCC8198 and its fermented foods still remained unclear. Here, the antioxidant and antimelanogenesis functions of *L. plantarum* CGMCC8198-fermented milk were investigated.

Materials and Methods

Chemicals and reagents Kojic acid, 3,4-dihydroxyphenylalanine (L-DOPA), mushroom tyrosinase, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-3-ethylbenzothylbenzothiazoline-6-sulphonic acid (ABTS), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). Antibodies against TYR, TRP-1, and TRP-2 were obtained from Abcam (Abcam, Cambridge, MA, USA.).

Cell Culture. B16F10 mouse melanoma cells (Shanghai institute of cell biology, cell bank, Chinese academy of sciences) were cultured in Dulbecco's Modified Eagle's Medium (DMEM;

Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; sijiqing, Hangzhou, China), penicillin (100 U/mL) and streptomycin (100 U/mL) at 37°C in humidified air with 5% CO₂.

Determination of the growth curve The growth curve of *L. plantarum* CGMCC8198 in milk was measured by the plate count method (Bevilacqua *et al.*, 1989). Briefly, *L. plantarum* CGMCC8198 was inoculated in pure milk (PM) with 10% of the inoculum amount and anaerobic cultured at 37°C. One hundred microliters of fermentation liquor were taken at 6 h, 12 h, 24 h, 36 h, 48 h, 72 h and 96h respectively, then diluted and coated to the plate uniformly. After being cultured for 36 h, the number of colonies was counted.

Sample preparation *L. plantarum* CGMCC8198 (Key Laboratory of Industrial Fermentation Microbiology of the Ministry of Education) was inoculated in milk and then anaerobic cultured for 48 h at 37°C. Fermented product were harvested followed by centrifugation at 12,000 × g, 4°C for 20 min to obtain the aqueous phase (FMS), and then filtrated with 0.22 μm membrane and store at -20°C. In order to further obtain the high molecular substance (HFMS) and the low molecular substance (LFMS) of FMS, the FMS was separated by ultrafiltration tube with 3 kD aperture by centrifugation at 7000 × g, 4°C for 10 min.

Cell viability assay. Cell viability was determined by MTT assay. After incubation of cells with samples for 48 h, the culture medium was removed and replaced with 5 mg/mL of MTT solution dissolved in sodium potassium phosphate buffer (pH 6.8) and incubated in culture conditions for an additional 4 h. Subsequently, the MTT solution was removed, 100 μL DMSO was added, and the absorbance of dissolved formazan crystals was determined at 490 nm using a microplate reader (Synergy4, Biotek, USA).

Measurement of melanin content Melanin content was measured as described previously with minor modification (Yoon *et al.*, 2016). B16F10 cells (1×10⁵) were seeded in 6-well tissue culture plates. After 12 h incubation, the medium was replaced with serum-free DMEM and cells were further incubated for 24 h. The medium was then replaced with DMEM containing various concentrations of samples. After incubation for 48 h, the medium was removed to an eppendorf tube and photographed. To extract intracellular melanin, cell pellets were washed twice with cold PBS and then dissolved in 1 N NaOH at 65°C for 30 min. The melanin content of the cell extracts was measured at 490 nm with a microplate reader (Synergy4, Biotek, USA).

Detection of cell-free tyrosinase activity Cell-free tyrosinase activity was measured using the method of Yagi with minor modification (Kim, H. R. *et al.*, 2015). Briefly, 40 μL of 10 mM L-dihydroxyphenylalanine (L-DOPA), 40 μL of 125 units of mushroom tyrosinase, 80 μL of 67 mM sodium potassium phosphate buffer (pH 6.8), and 40 μL of different concentrations of FMS were mixed. Kojic acid was used as a control. Following incubation at 37°C for 10 min, the amount of dopachrome formation was determined by measuring the absorbance at 490 nm.

Table 1. Primers used in this study

Gene	Primer sequences (5'-3')	Product length (bp)
β -actin	Forward: 5'-CGTTGACATCCGTAAAGACC-3' Reverse: 5'-GAAGGTGGACAGTGAGGC-3'	199
TYR	Forward: 5'-ACACCTGAGGGACCACTAT-3' Reverse: 5'-CATTGGCTTCTGGGTAAACT-3'	373
TRP-1	Forward: 5'-GCCACAAGGAGGTTAGAAGACA-3' Reverse: 5'-CCAGTAAGGAAGGGAGAAAAGAG-3'	264
TRP-2	Forward: 5'-AGAAGTTTGACAGCCCTCC-3' Reverse: 5'-CAAGTTGCTCTGCGGTTAG-3'	360
MITF	Forward: 5'-AACGGGAACAGCAACGAGC-3' Reverse: 5'-TCACCAGATCAGGCGAGCA-3'	162

Inhibition of the activity of mushroom tyrosinase was indicated by a reduction in absorbance of the FMS-treated sample versus the blank sample.

Analysis of intracellular activity of tyrosinase Intracellular tyrosinase activity was determined by measuring dopachrome formation of L-DOPA after reaction with the cell lysate. B16F10 cells (1×10^5) were seeded in 6-well tissue culture plates. After 12 h incubation, the medium was replaced with serum-free DMEM and cells were further incubated for 24 h. The medium was then replaced with DMEM containing various concentrations of FMS and incubated for 48 h. Cells were washed twice with cold PBS and lysed with phosphate buffer (pH 6.8) containing 1% Triton X-100. Cell lysates were clarified by centrifugation at $12,000 \times g$ for 10 min. Each lysate (90 μ L) was placed in a 96-well plate, and a 10 μ L aliquot of 2 mg/mL L-DOPA was then added to each well. After incubation at 37°C for 30 min, the absorbance was measured at 490 nm using a microplate reader (Synergy4, Biotek, USA).

Reverse-transcription polymerase chain reaction Reverse-transcription polymerase chain reaction (RT-PCR) analysis was carried out as described previously. Briefly, total cellular RNA was extracted from cultured cells with Trizol reagent (Invitrogen) and reverse-transcribed using M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. The thermal cycle profile was as follows: denaturation for 30 sec at 95°C, annealing for 45 sec at 54°C, and extension for 30 sec at 72°C. For semi-quantitative assessment of transcription levels, each PCR reaction was carried out for 28 cycles. PCR products were visualized on 2% agarose gels stained with ethidium bromide under UV transillumination. β -actin was used as an internal control to show equal loading of the cDNA samples. The PCR primer sequences were listed in table 1.

Western blotting Western Blotting was performed as described previously. The total protein of the cells was prepared using extraction buffer composed of PBS containing 0.5% Triton X-100, EDTA, phenylmethyl sulfonylfluoride (PMSF), the complete protease inhibitors (Roche). The concentration of each protein lysate was determined by BCA protein assay kit (Thermo). Equal amount of total protein was loaded on 12% sodium

dodecylsulfate polyacrylamide gel. Then samples were transferred to NC membranes and blocked for 60 min at room temperature in 5% skim milk powder (w/v) in PBS. The membranes were immune blotted with mouse monoclonal antibody of GAPDH (1:5000 dilution, Santa Cruz) and rabbit monoclonal antibody of TYR (1:1000 dilution, Abcam), TRP-1 (1:1000 dilution, Abcam), TRP-2 (1:5000 dilution, Abcam) and MITF (1:1000 dilution, Abcam) antibodies overnight at 4°C, and then incubated with IRDye-800 conjugated anti-mouse or anti-rabbit secondary antibodies (Li-COR) for 60 min at room temperature. The specific proteins were visualized by Odyssey Infrared Imaging System (Gene Company). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an internal control to show equal loading of the protein samples.

Immunocytochemistry assay Immunocytochemistry assays were performed as described previously. The cells after treatment were fixed in 4% paraformaldehyde for 15 min, and then blocked with normal goat serum for 30 min at room temperature. Then, rabbit monoclonal antibody of TYR (1:200 dilution, Abcam) were added and incubated in a humid chamber overnight. After being washed with phosphate buffered saline (PBS) thrice, cells were incubated with fluorescein isothiocyanate (FITC)-goat anti-rabbit IgG (Santa Cruz) for 1 h at 37°C. After being washed with PBS, the samples were observed under laser scanning confocal microscope (Olympus, Japan). DAPI stain (blue) highlights the total nuclei.

Evaluation of antioxidant activity The original method of Blois (Blois, 1960) was slightly modified and used for the determination of scavenging activity of DPPH free radical. FMS was diluted in methanol, ranging from 10% to 40%. Two hundreds microliters of 0.9 mM DPPH prepared in methanol and two hundreds microliters various concentrations of FMS was added to each tube. A control tube containing methanol was maintained. The reaction mixture was incubated in dark at 37°C for 30 min. The absorbance was measured at 514 nm. Besides, ABTS assay was also performed to further evaluate the radical scavenging activity of FMS. The monocation of ABTS^{•+} is generated by oxidation of ABTS with potassium persulfate. The ABTS^{•+} was produced by

allowing 7 mM ABTS stock solution to react with 2.45 mM potassium persulfate (final concentration) in the dark at room temperature for 14–16 h before use. The ABTS^{•+} solution was diluted with phosphate buffered saline (PBS, pH 7.4) to an absorbance value of 0.70 (± 0.02) at 734 nm. Two hundreds microliters of this solution was added into 200 μ L of FMS or standard diluted in methanol and make concentrations of the sample ranged from 3% to 12%. The reaction mixture was incubated in dark at 37°C for 30 min. Absorbance was measured at 734 nm. The radical scavenging activity was calculated as follows:

Radical scavenging activity (%) = [(Absorbance control – Absorbance test)/Absorbance control] $\times$ 100.

Statistical analysis Data were analyzed with the Graphpad Prism program. Experimental groups were normalized to control groups and analyzed with t-test. *P* value < 0.05 or < 0.01 was indicated by * or **, respectively. Data were presented as mean $\pm$ SD.

Results

Growth of *L. plantarum* CGMCC8198 in milk First of all, to detect whether *L. plantarum* CGMCC8198 can growth in milk, the growth curve was detected using plate count method. As shown in Fig1, the strain was able to grow well in milk, it entered the logarithmic growth phase at about 24 h, reached the plateau phase and got the maximum cell number as 3.14×10^8 at about 72 h (Fig. 1). These result suggested that *L. plantarum* CGMCC8198 could be used as a starter for the milk fermentation.

***L. plantarum* CGMCC8198-fermented milk decreased melanogenesis in B16F10 cells but not affected the survival of cells** To investigate whether FMS and, PM could inhibit melanogenesis, the melanin content of B16F10 cells was measured after treatment with FMS and PM. As a positive control, kojic acid, a well-known tyrosinase inhibitory, was tested simultaneously. Cells were exposed to the FMS, PM (5%, 10%, 15% and 20%) or 800 μ M of kojic acid for 48 h, and then the amounts of melanin secreted into the medium and retained in intracellular spaces were measured individually. As shown in Fig. 2A and 2B, after being treated by either FMS or PM, the medium color of melanocytes became lighter than that of untreated cells, and the intracellular melanin content were also reduced. Furthermore, it is noteworthy that the melanogenesis-inhibitory activity of FMS is much higher than that of PM, indicating that some melanogenesis-inhibitory active substrates might be biotransformed and increased during the milk fermentation by *L. plantarum* CGMCC8198. Therefore, we chose the FMS instead of PM as the major study object in the following research.

In order to evaluate whether the reduction of melanin induced by FMS is caused by the cytotoxicity of FMS or not, MTT assay was performed. As shown in Fig. 2C, cell death was not observed after treatment with FMS at a concentration range of 5 – 20%, at which FMS has exhibited significant melanogenesis-inhibitory

Fig. 1. The growth curve of *L. plantarum* CGMCC8198 in milk.

activity. This result indicated that FMS might inhibit melanin synthesis without cytotoxic effects on cells.

***L. plantarum* CGMCC8198-fermented milk inhibits intracellular but not extracellular tyrosinase activity** Next, the effect of FMS on the intracellular activity of tyrosinase in B16F10 melanoma cells was analyzed. As shown in Fig. 3A, FMS could dose-dependently decrease intracellular activity of tyrosinase in B16F10 cells, and its effect is higher than 800 μ M kojic acid when the concentration is over 15%. However, when we examined the effect of FMS on the activity of tyrosinase in a cell-free system, it showed that FMS had no direct inhibitory effect on extracellular tyrosinase activity (Fig. 3B), whereas kojic acid could still suppress the tyrosinase activity in a dose dependent manner (Fig. 3C). These results indicated that FMS might reduce the content of tyrosinase in melanoma cells rather than directly inhibit the enzyme activity.

***L. plantarum* CGMCC8198-fermented milk downregulates tyrosinase family through MITF signal transduction pathway** Furthermore, to test whether FMS could regulate the transcription and expression of melanogenesis-related genes, B16F10 melanoma cells were treated with FMS (5%, 10%, 15% and 20%) for 48 h, and then the mRNA and protein level of TYR, TRP-1, TRP-2 and MITF were assayed by RT-PCR, western blotting and immunocytochemistry. As shown in Fig. 4 and Fig. 5, compared with untreated control cells, FMS treatment significantly reduced both mRNA and protein levels of TYR, TRP-1, TRP-2 and MITF in a dose-dependent manner. In the experiments of RT-PCR, the inhibitory effect of FMS on TYR was the most obvious. When the concentration of FMS was 20%, the inhibition rate on TYR reached 97.97%, and the inhibition rate for TRP-1, TRP-2 and MITF were 63.49%, 60.89%, and 63.75% respectively (Fig. 4D and 5D). The similar results were also observed in the Western Blot assay (Fig. 4E and 5E) and the immunofluorescence assay (Fig. 4C). These results suggested that FMS might inhibit melanogenesis via suppressing the transcription and expression of tyrosinase family genes (especially TYR), key enzymes for the biosynthesis of melanin, through MITF signal transduction pathway.

Fig. 2. Effect of *L. plantarum* CGMCC8198-fermented milk on melanin production and cell viability of B16F10 cells. B16F10 cells were incubated with various concentrations of the FMS, PM (5%, 10%, 15% and 20%) or 800 μ M of kojic acid, the positive control, for 48 h. The melanin content was detected by measuring the absorbance at 490 nm using a microplate reader (A) and the color of cell culture medium was photographed (B). Besides, the cell viability measured by MTT assay (C). Data are mean $\pm$ standard deviation. * P < 0.05, ** P < 0.01 compared with the vehicle-treated group (n = 3)

The melanogenesis-inhibitory and antioxidant activities of *L. plantarum* CGMCC8198-fermented milk are mainly depended on heat-resistant macromolecule substances. Finally, in order to detect what kind of ingredient in FMS plays main roles in the

Fig. 3. Effect of *L. plantarum* CGMCC8198-fermented milk on intracellular tyrosinase activity and the cell-free tyrosinase activity. B16F10 cells were incubated with various concentrations of the FMS (5%, 10%, 15% and 20%) or 800 μ M of kojic acid, the positive control, for 48 h, and the intracellular tyrosinase activity was evaluated by L-DOPA oxidation assay (A). Besides, the cell-free tyrosinase activity of kojic acid (C) and FMS (B) was also determined using mushroom tyrosinase. Data are mean $\pm$ standard deviation. * P < 0.05, ** P < 0.01 compared with the vehicle-treated group (n = 3)

inhibition of melanogenesis, the FMS was separated into high-molecular substances (HFMS, > 3 kD) and low-molecular substances (LFMS, < 3 kD) by ultrafiltration. After detecting the melanogenesis-inhibitory effects of HFMS and LFMS, we found

Fig. 4. Inhibitory effects of FMS on transcription and expression of key enzymes of melanin biosynthesis. B16F10 cells were incubated with various concentrations of the FMS (5%, 10%, 15% and 20%) or kojic acid (800 μ M), the positive control, for 48h. RT-PCR assay were performed to estimate mRNA expression levels of tyrosinase (TYR), tyrosinase related protein-1 (TRP-1) and tyrosinase related protein-2 (TRP-2). β -actin was also detected as control (A). Protein level of tyrosinase family was analyzed by Western Blotting, and protein loading amounts were confirmed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (B). Besides, the expression of TYR was further estimated by immunocytochemistry again. DAPI stained the nuclear in blue, and TYR stained in green (C). In addition, the mRNA and protein levels of TYR, TRP-1 and TRP-2 in the RT-PCR and Western Blotting assay were semi-quantified using β -actin (D) or GAPDH (E) as the internal control by Image J software. Values represent the mean $\pm$ SD for three independent experiments. * P < 0.05, ** P < 0.01 compared with the vehicle-treated group (n = 3)

that FMS, HFMS and LFMS reduced intracellular melanin by 29.82%, 41.24% and 22.99% (Fig. 5A), respectively, at same 15% dosage. Besides, the suppression effects of HFMS on the transcription and expression of MITF were also much higher than those of FMS and LFMS. At the transcription level, the inhibition rate of HFMS was 86.08%, while FMS and LFMS were only 63.75% and 29.35%, respectively (Fig. 5B, 5D). As for protein expression level, the inhibition rate of HFMS was 87.68%, whereas FMS and LFMS only exhibited 72.78% and 69.39%, respectively.

(Fig. 5C, 5E). These data indicated that the melanogenesis-inhibitory activity of *L. plantarum* CGMCC8198-fermented milk is mainly depended on macromolecule substances. In order to further investigate whether the macromolecule bioactive substances might be proteins or polysaccharides, FMS, LFMS and HFMS were treated at 85°C for 30 min to denature and inactivate the protein in the samples, and then the melanogenesis-inhibitory assay was performed again. As shown in Fig. 6A, the activity of FMS, LFMS and HFMS did not exhibit any significant change after the heat

Fig. 5. The effect of various molecular substances of FMS on melanogenesis and the expression of MITF. FMS was separated into high-molecular substances (HFMS, > 3 kD) and low-molecular substances (LFMS, < 3 kD) by ultrafiltration. Effect of FMS, LFMS and HFMS on intracellular melanin content, at same 15% dosage, were detected (A). The mRNA levels of MITF in cells treated by FMS, LFMS and HFMS were detected by RT-PCR assay, and β -actin was also detected as control (B). The protein levels of MITF were analyzed by Western Blotting. Protein loading amounts were confirmed by GAPDH expression (C). The transcription and expression of MITF were also semi-quantified using Image J software (D & E). Values represent the mean $\pm$ SD for three independent experiments. * P < 0.05, ** P < 0.01 compared with the vehicle-treated group (n = 3).

treatment. Besides, we also detected the antioxidant activity of these samples, and similar results were also observed. Both ABTS and DPPH assay showed that the antioxidant activity of HFMS was higher than those of FMS and LFMS, and heat treatment could hardly affect the activity of these samples (Fig. 6B-6G). Taken together, to our opinion, the key active ingredients in FMS might be polysaccharides, and further identification studies should be performed in the future.

Discussion

Melanocytes protect the body from UV radiation by producing melanin and eliminating reactive oxygen species (ROS). However, abnormal accumulation of melanin causes esthetic problems and hyperpigmentation disorders, such as freckles, chloasma, lentigo, liver spots, and melisma (Ahn *et al.*, 2006; Unver *et al.*, 2006). Although kojic acid, arbutin and several skin whitening products were widely used in cosmetics, their adverse reactions were

Fig. 6. Effects of heat treatment on the melanogenesis-inhibitory and antioxidant activities of *L. plantarum* CGMCC8198-fermented milk. FMS, LFMS and HFMS were treated at 85°C for 30 min, and then the intracellular melanin content after being treated with heated or non-heated FMS, LFMS and HFMS at same 15% dosage, were detected (A). Besides, the antioxidant activities of these samples were analyzed using ABTS⁺ assay (B, D, and F) and DPPH scavenging assay (C, E, G).

disturbing. For example, kojic acid had carcinogenic possibility and arbutin exhibited weak photostability (Masse *et al.*, 2001; Suh *et al.*, 2009; Yang *et al.*, 2013). Therefore, safer and more effective skin lightening agents were still needed to be sought.

In the present study, our results suggested that the fermented milk supernatant (FMS) prepared by *L. plantarum* CGMCC8198, a novel probiotics strain isolated in our previous research, exhibited more significantly melanin suppressed effect than pure milk (PM). The melanogenesis-inhibitory activity of *L. plantarum* CGMCC8198-fermented milk is mainly due to its transcriptional repression effects on tyrosinase family through MITF signal transduction pathway. Plenty of evidence has demonstrated that the initial step of melanogenesis process is the conversion of L-tyrosine to 3, 4-dihydroxyphenylalanine (L-DOPA) and then the oxidation of L-DOPA yields dopaquinone by tyrosinase (Kameyama *et al.*, 1995). TRP-2 catalyzes the rearrangement of DOPA chrome to 5, 6-dihydroxyindole-2-carboxylic acid (DHICA) (Fang *et al.*, 2001) and TRP-1 catalyses the oxidation of DHICA to indole-5, 6-quinone-2-carboxylic acid (Fang & Setaluri, 1999). MITF is regarded as an essential transcriptional regulator of tyrosinase family and plays important roles in melanogenesis, pigmentation and melanocyte differentiation. Pigmentation defects in skin, eyes and hair have been observed in patients suffering from mutations of MITF (Lin *et al.*, 2002; Wellbrock & Arozarena, 2015). Previous studies have shown that *L. rhamnosus*/*Saccharomyces cerevisiae*-fermented rice bran could inhibit the melanogenesis via suppression of MITF (Chung *et al.*, 2009). Similarly, in our study, the decreased of MITF may play an important role in the inhibition of melanogenesis by *L. plantarum* CGMCC8198-fermented milk.

Accumulating studies have also shown probiotics could act as ROS scavengers and antioxidants. Kodali and his colleagues reported that an extracellular polysaccharide produced by *Bacillus coagulans* RK-02 had significant antioxidant and free radical scavenging activities (Kodali & Sen, 2008). Tang *et al.* found that *L. plantarum* MA2 colonizes and survives in the murine intestinal tract to exert its antioxidative effects (Tang *et al.*, 2016). Jiang and his cooperators demonstrated that the Aloe fermentation supernatant fermented by *L. plantarum* HM218749.1 had very strong scavenging capacities of the DPPH, O₂⁻, ·OH, and Fe²⁺ chelation and reducing powers (Jiang *et al.*, 2016). Consistent with these reports, our data also suggested that *L. plantarum* CGMCC8198-fermented milk had markedly antioxidant effect, which was also of value in the application as cosmetic or medical whitening agent.

Furthermore, our data showed that both melanogenesis-inhibitory activity and antioxidant activity of FMS, especially the high-molecular substance in FMS (HFMS, > 3 kD), were much better than those of pure milk, and these activities were not affected by heat treatment. Therefore, to our opinion, the key active substrates of *L. plantarum* CGMCC8198-fermented milk might be polysaccharides rather than proteins. Similarly, previous studies

also showed that exopolysaccharides of lactobacillus have antioxidant activity and the biosynthesis of exopolysaccharides by lactobacillus could be significantly enhanced in whey-based media (Liu *et al.*, 2011)

In summary, this study demonstrated that *L. plantarum* CGMCC8198-fermented milk exhibited melanogenesis-inhibitory and antioxidant effects. The findings might provide a natural candidate for the development of skin whitening agent and treatment for hyperpigmentation disorders

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Author Contribution

Xue-Gang Luo and Tong-Cun Zhang designed the experiments. Cai-Jiao Zhang, Li-Juan Pan and Wen-Han Lu performed the experiments. Xue-Gang Luo and Cai-Jiao Zhang analyzed data. Hao Zhou and Nan Wang contributed reagents, materials and tools. Xue-Gang Luo and Cai-Jiao Zhang wrote the paper.

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