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ORIGINAL ARTICLE

Skin resistance to UVB-induced oxidative stress and hyperpigmentation by the topical use of *Lactobacillus helveticus* NS8-fermented milk supernatant

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Keywords
antioxidant capacity, *Lactobacillus helveticus* NS8-FS, melanogenesis, skin, UV radiation.

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Abstract

Aims: In this study, we investigated the preventive properties of the supernatant of *Lactobacillus helveticus* NS8-fermented milk (NS8-FS) against UV light-induced skin oxidative damage and hyperpigmentation.
Methods and Results: NS8-FS exhibited significant radical scavenging activity in tests with $ABST^{+}$ and DPPH scavenging methods, and as well strongly inhibited 3-morpholinosydnonimine (Sin-1)-induced ROS generation in HaCat keratinocytes. Unexpectedly, NS8-FS was found to inhibit melanin production in B16F10 melanoma cells and to exhibit inhibitory effects both to the enzymatic activity of tyrosinase (TYR) and the expression of proteins required for melanin synthesis. In SKH-1 hairless mice, topical application of NS8-FS alleviated UVB-induced skin photodamage, including the improvement of the appearance of epidermal thickness, transepidennal water loss and lipid peroxidation levels. In the tanning guinea pig model, the whitening effect of NS8-FS was demonstrated using Masson-Fontana staining and TYR staining. Furthermore, NS8-FS was shown to stimulate the nuclear translocation and activation of the Nrf2 protein, along with recovery of antioxidant enzyme activities.
Conclusion: NS8-FS exhibits the protective capacities against UV light-induced skin oxidative damage and hyperpigmentation.
Significance and Impact of the Study: Our findings indicate the potential of cell-free fermented products of lactic acid bacteria in topical photoprotection.

Introduction

Solar ultraviolet radiation (mainly UVA and UVB) has been widely considered a major causative factor in the initiation of several skin disorders, such as erythema, dryness, pigment abnormalities, inflammation, degenerative ageing changes and skin cancers (Armstrong and Kricker 2001; Ichihashi *et al.* 2003; Amaro-Ortiz *et al.* 2014). Continuous exposure to UV radiation diminishes the efficiency of cellular antioxidant enzyme systems and promotes the accumulation of free radicals and reactive oxygen species (ROS) (Pillai *et al.* 2005). The adaptive tanning response involved of activating biosynthetic enzymes tyrosinase (TYR) and related proteins protects epidermal cells from initial UV radiation (Wasmeier *et al.* 2008). However, prolonged activation of melanogenesis upon exposure to UVA or UVB independently leads to hyperpigmentation of skin (Videira *et al.* 2013). In this context, encouraging photoprotection is the leading skin care health strategy.

Skin protection commonly involves sunscreen ingredients exerting antioxidant and anti-melanogenesis benefits

during solar exposure, derived from synthetic and natural products, such as vitamin C, carotenoids and polyphenols (Nichols and Katiyar 2010; Fernandez-Garcia 2014). In fact, selected strains of *Lactobacillus* and *Bifidobacterium* have been reported to show a range of health-promoting functions, some of which are based on its antioxidant properties, improving total antioxidant status in humans (Songisepp *et al.* 2005; Virtanen *et al.* 2007). The beneficial bacteria hold promise not only for the intestinal tract but also distant niches and organs, including the skin. According to some clinical trials, the oral uptake of specific probiotic strains or fermented milk was found to improve the physiologic conditions of normal skin, such as increasing surface moisture retention, reducing transepidermal water loss (TEWL) and alleviating wrinkling and erythema (Kimoto-Nira *et al.* 2014; Lee *et al.* 2015). In particular, the pre-administration of some probiotics or bacterial lysates showed resistance to UV-induced oxidative stress and consequently protected the skin against photodamage in murine models (Weill *et al.* 2013; Kim *et al.* 2014). The exact mechanisms underlying these effects are poorly understood. Quite a number of probiotic strains were capable of producing antioxidant molecules, such as glutathione, during gastrointestinal digestion or industrial processing (Pophaly *et al.* 2012). Moreover, a higher proportion of low-molecular weight polypeptides in lactic acid bacteria (LAB) fermented milk elicited the high scavenging activity of the fermented products (Virtanen *et al.* 2007). However, few investigations have been carried out to assess such protective properties and the targeted pathways of cell-free probiotic cultures. Only the culture filtrate of *Bifidobacterium* was shown to have antioxidant activity and an inhibitory effect on melanogenesis *in vitro* (Huang and Chang 2012). Considering the accepted safety of probiotic fermented milk, it is feasible to use fermented products in cosmeceutical therapeutics for skin care.

Lactobacillus helveticus NS8, isolated from Mongolian koumiss, has shown good probiotic functions in our prior studies, including colitis-attenuating activities (Rong *et al.* 2015). In this study, NS8-fermented milk filtrate was first observed to have comparable radical scavenging and ROS inhibitory capacities *in vitro* tests and, unexpectedly, was found to inhibit melanogenesis in melanoma cells. We wondered whether the topical application of this filtrate could effectively and safely exert protection against UV light-induced skin damage. Thus, we assessed this possibility in UVB-irradiated animal experiments, by measuring the changes of the surface physiological conditions and enzymatic activities of the skin's antioxidant defences. We found that NS8-fermented milk filtrate administered topically can effectively prevent chronic UVB-induced oxidative damage and melanogenesis, in which the modulation of nuclear factor erythroid-2-related factor-2 (Nrf2) activity seems to be the underlying mechanism.

Materials and methods

The preparation of lactobacilli-fermented milk filtrate

Lactobacillus helveticus NS8, obtained from fermented koumiss, was stored at −80°C in de Man, Rogosa and Sharpe (MRS) medium supplemented with 15% (v/v) glycerol. The strain was revitalized in MRS broth at 37°C for 48 h before use. For the preparation of the NS8-fermented milk supernatant, bacterial cells were transferred to 12% skim milk (m/v) and cultured at 37°C for another 12 h. Supernatant (NS8-FS) was obtained by centrifugation at 10 000 ***g*** for 10 min at 4°C and filtration through a 0·2-μm filter. The pH of the milk supernatant was adjusted to 4·6, and it was kept at 4°C until use.

Cells and treatment

Human HaCat keratinocyte cells and mouse B16F10 melanoma cells were obtained from the American Type Culture Collection. HaCat cells were grown in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Logan, UT), supplemented with 10% (v/v) foetal bovine serum (FBS; HyClone). Mouse B16F10 melanoma cells were cultured in RPMI Medium 1640 (HyClone) with 10% FBS. Both cultures were added to 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin and kept at 37°C in a humidified 5% CO_2 atmosphere. For the *in vitro* tests, the NS8-fermented milk supernatants at various concentrations (v/v), as shown in the figures, were added to the cellplates when the cultures reached 70–80% confluence. The conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used for the cell viability assay, following the NS8-FS treatment for 24 h (Berridge *et al.* 2005).

Antioxidant activities assay *in vitro*

The antioxidant activities of NS8-FS were first determined by measuring the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^+$) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging abilities. For the $ABTS^+$ scavenging assay, the tests were carried out by following the instructions from the Total Antioxidant Capacity Assay Kit (Beyotime Technology, Shanghai, China). For the DPPH scavenging assay, the tests were performed using the method previously described (Sánchez-Moreno *et al.* 1998). Briefly, NS8-FS of various

concentrations was added to the 60 mmol l^{-1} DPPH solutions (Sigma-Aldrich, St. Louis, MO). The reduced form of DPPH was quantified as the decrease in absorbance at 517 nm using Varioskan® Flash microplate reader (Thermo Scientific, Rockford, IL).

At the intracellular level, HaCat cells were treated with NS8-FS for 2 h, and then incubated in the probe H_2DCFDA (20 μmol l^{-1}; Sigma-Aldrich) containing serum- and phenol red-free DMEM for 45 min. Then, the medium was replaced with 100 μl Sin-1 (10 μmol l^{-1}) as the ROS stimulant and incubated for another 1 h. Fluorescence was recorded while excitation was read at 485 nm and emission at 535 nm.

Melanin content and tyrosinase activity assay *in vitro*

Melanin content was determined using a modification of the method described (Tsuboi *et al.* 1998). Mouse B16F10 melanoma cells were treated with NS8-FS at various concentrations for 24 h. Then, the pellets of 2×10^6 cells were collected and solubilized in 100 μl 1 mol l^{-1} NaOH at 60°C for 1 h. The absorbance of the solutes was assayed at 405 nm. Under the same treatment, the intracellular TYR activity of the B16F10 cells was estimated by measuring the L-DOPA oxidation capacity (Yang *et al.* 2006). The melanoma cells were collected and lysed with 1× PBS containing 1% Triton X-100 and 0·1 mmol l^{-1} phenylmethylsulfonyl fluoride (Amesco, Solon, OH). Cellular extracts (100 μl) were mixed with freshly prepared 100 μl L-DOPA (0·1%; Sigma-Aldrich) and incubated at 37°C for 30 min. The products of the reaction were measured by reading the absorbance at 490 nm. In addition, mushroom TYR was used as the other enzyme source *in vitro* (Bilodeau *et al.* 2001). A total volume of 200 μl of mixture containing NF8-FS and 5 mmol l^{-1} L-DAPA was added to a 96-well microplate containing 10 μl of mushroom TYR (1000 U; Sigma-Aldrich). Following incubation at 37°C for 30 min, the amount of dopachrome was measured by reading the microplate at 490 nm.

UVB-induced animal models

Female SKH-1 hairless mice (8 weeks of age, 10 each group) were obtained from the Laboratory Animal Center of Hangzhou Normal University. The UVB irradiation was performed as described with a modification (Matsuda *et al.* 2013). The animals were exposed to a UVB lamp (peak emission at 313 nm; Q-Lab, Westlake, OH) three times a week. The energy radiated was measured using a radiometer with a UVX-31 sensor (UVP, San Gabriel, CA). The initial dose of UVB was set at 36 mJ cm^{-2} (the minimum erythema dose (MED) for SKH-1 mice), which was subsequently increased weekly by 1/3 MED until the total irradiation doses reached 1·8 J cm^{-2} (8 weeks). The mice received NS8-FS (200 μl to 2 × 2 cm^2 dorsal skin) daily via a topical spray, typically 2 h before each irradiation session.

The ultraviolet radiated guinea pig model is usually used to mimic human pigmentation, as its skin contains active epidermal melanocytes located in the basal layer in a similar pattern to human skin (Bolognia *et al.* 1990). So for the anti-melanogenesis study, 10 brown guinea pigs (female, 300–350 g) were purchased from the Laboratory Animal Research Center of Zhejiang Chinese Medical University. The guinea pigs were placed under deep anaesthesia with chloral hydrate (250 mg kg^{-1}), and three dorsal skin sections (2 × 2 cm^2) were evenly shaved with infant clippers for pre-treatment with NS8-FS, or Kojic acid (an inhibitor of TYR, 5%, m/v) or no treatment before the first UVB irradiation session. The irradiation procedure and topical administration were similar to the experiments conducted in SKH-1 mice, except the initial dose of UVB was set at 120 mJ cm^{-2} and lasted until distinct pigmentation appeared (3 weeks). After the procedure, a fourth dorsal skin section without irradiation was shaved as blank control.

Skin physiological properties assay

After the final treatments, the physiological parameters on the live animal skin, including TEWL, skin pH, skin hydration, melanin and erythema, were assessed with a Multiprobe Adapter System (MPA5; Courage-Khazaka, Cologne, Germany). Different probes were used in the examination according to the manufacturer's instructions, such as the Tewameter® TM300 probe for measuring TEWL, the Corneometer® CM825 for measuring hydration values, the Skin-pH meter® PH905 for measuring skin pH, and the Mexameter® MX 18 probe for measuring melanin and erythema. The results are expressed as the medium indexes with general units obtained by Mexameter software.

Histological and immunohistochemical analyses

The dorsal skin was rapidly separated after the animals were euthanized with carbon dioxide. Histological analysis was performed on haematoxylin/eosin (H&E)-stained skin samples fixed in 4% paraformaldehyde and embedded in paraffin. The thickness of the epidermis in the samples stained with H&E was also measured by selecting four different microscopic fields per sample. The guinea pig skin melanin was stained by using a Masson-Fontana staining kit according to the protocol supplied by the manufacturer (Abcam, Cambridge, MA). For the

immunohistochemical TYR staining, the skin sections were incubated with anti-TYR antibody (1 : 100; Abcam) followed by horseradish peroxidise-conjugated anti-rabbit IgG (1 : 1000; Abcam). All the microscopic images were captured with a Pannoramic Scanner and were analysed using the Pannoramic Viewer (PerkinElmer, Waltham, MA) software.

Antioxidant enzyme activities assay

Skin samples from the −80°C storage were homogenized at 4°C, in 50 mmol l^{-1} Tris-HCl (pH 7·5) with 5 mmol l^{-1} EDTA. The homogenates were centrifuged at 10 000 ***g*** for 15 min, at 4°C. The supernatants were directly used for the determination of enzyme activities with the Catalase (CAT) Assay Kit, the Superoxide Dismutase (SOD) Assay Kit and the Total Glutathione Peroxidase (GPX) Assay Kit (Beyotime Technology), following the manufacturers' protocols. Also, the skin sample lipid peroxidation levels were measured with the Lipid Peroxidation MDA Assay Kit (Beyotime Technology).

Western blotting

To prepare cytosolic and nuclear extracts from cultured HaCaT cells, the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) were used. For total cellular protein extraction, the cells were lysed in a buffer (pH 7·4) containing 150 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA, 10 mmol l^{-1} Tris-HCl and a protease inhibitor cocktail (Thermo Scientific). The proteins were separated by SDS-PAGE and then were transferred to nitrocellulose membranes. The protein-specific antibodies used as immunoreactive probes were anti-TYR (1 : 1000; Santa Cruz Biotechnology, Dallas, TX), anti-MITF (1 : 1000; Abcam), anti-TRP-1 (1 : 1000; Santa Cruz Biotechnology), anti-TRP-2 (1 : 1000; Santa Cruz Biotechnology), anti-Nrf2 (1 : 1000; Abcam), anti-β-Actin (1 : 2000; Santa Cruz Biotechnology) and anti-Histone 3 (1 : 1000; Abcam). The immunoreactive proteins were detected with the Odyssey® CLx Infrared Imaging System (Li-COR Biosciences, Lincoln, NE).

Cell immunofluorescence assay

To detect the cellular Nrf2 protein immunofluorescence, HaCat cells were inoculated onto glass coverslips in a six-well plate and were exposed to NS8-FS for 24 h. Then, the cells were fixed in 4% paraformaldehyde in PBS buffer, permeabilized by 0·3% Triton X-100, blocked using 3% bovine serum albumin for 1 h, and stained overnight in primary anti-Nrf2 antibody (1 : 500; Abcam). The next day, the cells were incubated for 1 h with anti-rabbit secondary antibody conjugated with FITC (1 : 1000; BD Biosciences, Franklin Lakes, NJ). The cells were counterstained with DAPI (Sigma-Aldrich) before the cell morphology was captured by a fluorescence microscope (Zeiss, Oberkochen, Germany).

Ethics statement

The Animal Care and Ethics Committee at Hangzhou Normal University approved all of the animal experiments in our study. All animals were fed with standard diet and tap water and kept in a specific-pathogen-free environment at 23°C with 12-h light/12-h dark cycle. During the experiments, all surgery was performed under anaesthesia, and all efforts were made to minimize suffering.

Statistical analysis

Comparisons between groups were conducted using one-way ANOVA. Data are expressed as the mean ± SD or mean ± SEM. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{**}P < 0{\cdot}001$. Each experiment was repeated at least three times.

Results

NS8-FS inhibited Sin-1 induced ROS production in HaCat cells without affecting cell growth

In the tube tests, NS8-FS clearly had antioxidant abilities such as scavenging free radicals $ABTS^{+}$ and DPPH in a dose-dependent manner, comparable to 20 μmol l^{-1} of vitamin C when the concentration of NS8-FS rose to 10% (Fig. 1a,b). At the intracellular level, a proliferation assay was first used to assess the cytotoxicity of NS8-FS. It had little effects on the growth of human HaCat keratinocyte cells when the concentration reached 10% (Fig. 1c). Similar results were observed when using other cell lines, such as melanoma B16F10 cells or hepatoma HepG2 cells (data not shown). Then, we investigated whether NS8-FS could inhibit the generation of ROS induced by Sin-1, a typical ROS-inducing compound. As expected, Sin-1 significantly increased ROS levels compared with those in the control cells (Fig. 1d). In addition, pre-incubation with NS8-FS at a concentration of 10% for 2 h could notably suppress the fluorescent ROS production in HaCat cells (nearly 50% inhibition) (Fig. 1d). These results suggest that NS8-FS has good antioxidant capacities *in vitro*.

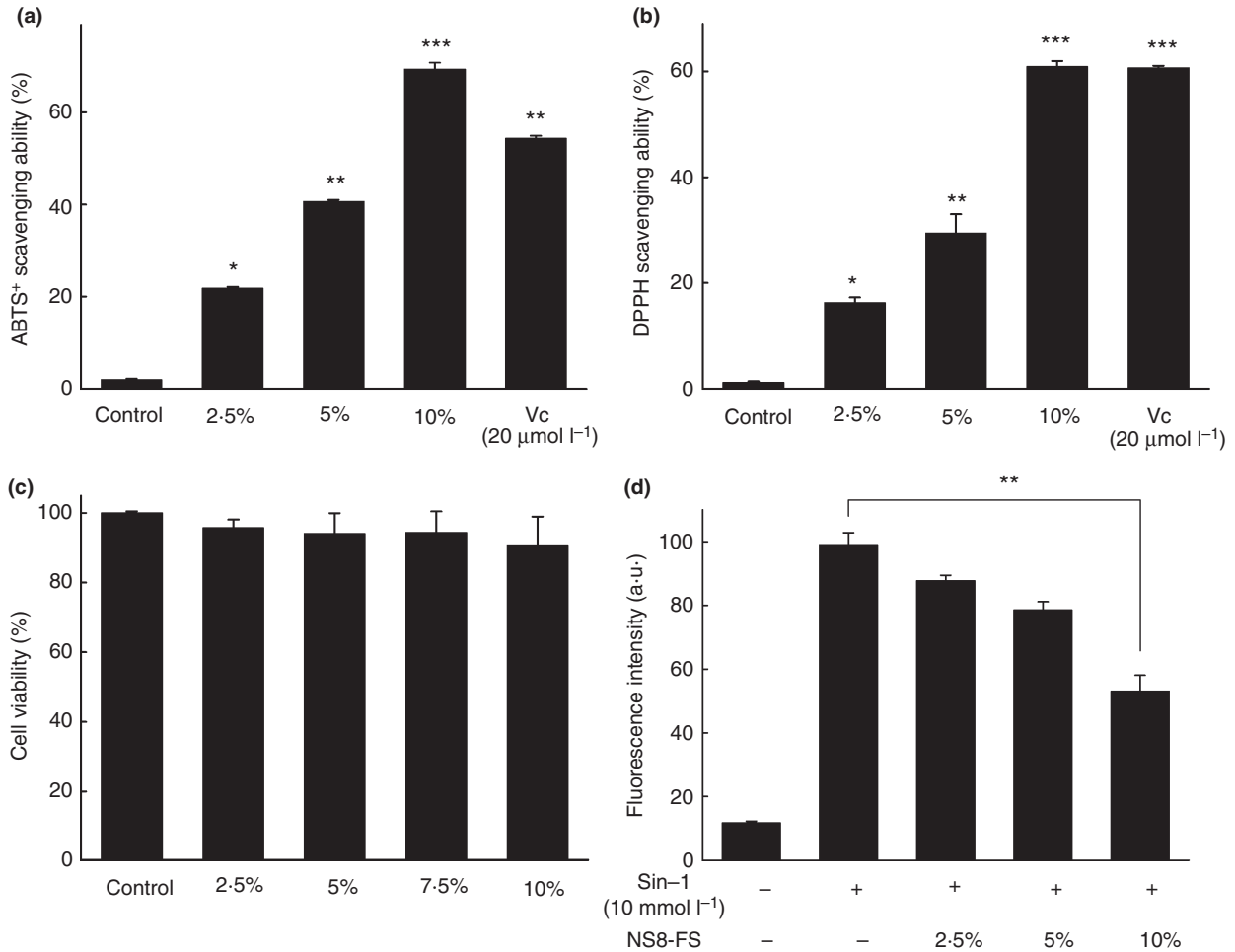

Figure 1 The antioxidant capacities of NS8-FS *in vitro*. The radical scavenging activities of NS8-FS were measured by both the ABTS$^+$ scavenging assay (a) and the DPPH scavenging assay (b). Vitamin C was used as a positive radical scavenger. In cultured HaCat cells, cell viability was measured using the MTT assay (c). The fluorescent DCFDA assay was used to determine the ROS-reducing level of NS8-FS (d). The cells were pre-treated with NS8-FS for 2 h and were further treated with 10 μmol l^{-1} Sin-1 for another 1 h. All the data are expressed as the mean ± SD from triplicate experiments. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.

NS8-FS inhibited melanogenesis by inhibiting the enzymatic activity and expression of tyrosinase in B16F10 cells

When we tested the antioxidant activities of NS8-FS in the cell cultures described above, we surprisingly observed that the colour of B16F10 cells lightened after treatment with NS8-FS (Fig. 2a). By measuring the melanin content and TYR activities in B16F10 cells, we found that NS8-FS did suppress the melanogenesis of B16F10 cells in a dose-dependent manner. NS8-FS, at a concentration of 10%, reduced the melanin content by nearly 40%, while similarly inhibiting the intracellular TYR activity (Fig. 2b,c). Moreover, to confirm the TYR inhibition, the activities of tyrosinase from mushroom after treatment with NS8-FS at various concentrations (or not) were also detected. NS8-FS at a concentration of 10% inhibited the enzymatic activities of mushroom TYR to the same extent as Kojic acid (0·2 mmol l^{-1}), a positive anti-melanin compound (Fig. 2d).

Although the data indicated that the decline in the melanin content of B16F10 cells treated with NS8-FS resulted from the inhibition of TYR activity, the expression of TYR was also measured in our tests. NS8-FS significantly downregulated the protein expression of tyrosinase in B16F10 cells as well as other key factors for melanosome biogenesis, such as MITF, TRP-1 and TRP-2 (Fig. 2e). In the quantitative real-time PCR assay, the transcriptional expression levels of these genes of the TYR family were also reduced in the presence of NS8-FS (supporting information Figure S1). Thus, both the enzymatic inhibition and protein expression downregulation

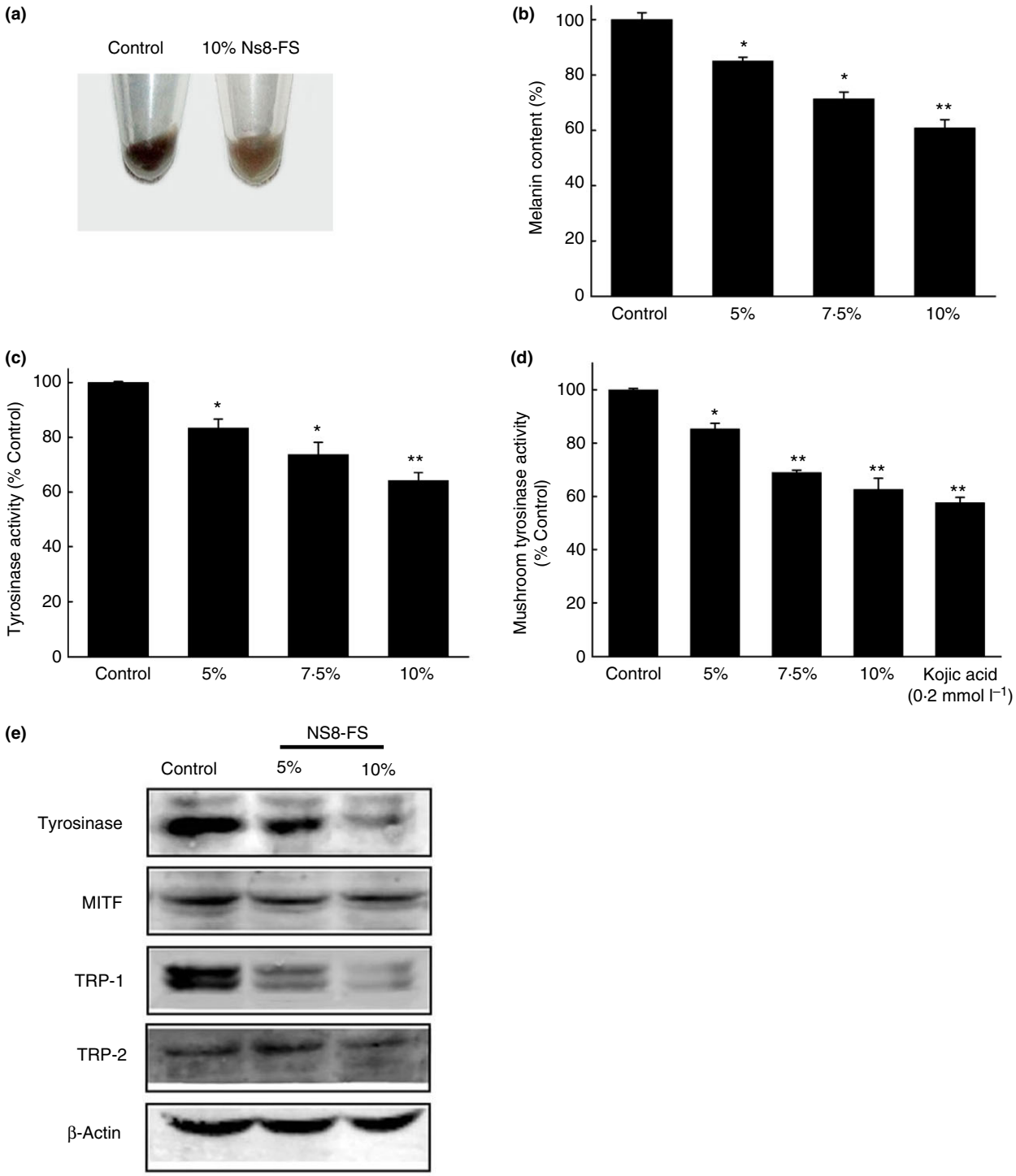

Figure 2 The anti-melanogenesis effects of NS8-FS *in vitro*. B16F10 cells were treated with NS8-FS for 24 h and the colour of the cell precipitate is presented as pictures (a). Intracellular melanin content was quantified spectrophotometrically (b). The treated cells were also lysed to obtain the cell-free extracts, and the intracellular tyrosinase activities were measured (c). In addition, mushroom tyrosinase activities were assayed in a tube test when Kojic acid was used as a positive control (d). The effect of NS8-FS on the expression of the tyrosinase pathway was further investigated. B16F10 cells were treated as described above. Total protein was extracted and subjected to Western blotting using anti-tyrosinase/MITF/TRP-1/TRP-2/β-Actin antibodies (e). The results of (b–d) are expressed as the mean ± SD from triplicate experiments. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ *vs* the untreated group. [Colour figure can be viewed at wileyonlinelibrary.com]

of cellular TYR are responsible for the anti-melanogenesis effects of NS8-FS.

NS8-FS prevented the UVB-induced photodamage and decrease of antioxidant defence in the skin of hairless mice

On the basis of the *in vitro* results, the protective effectiveness of NS8-FS for the skin was further assayed using UVB-irradiated animal models. First, we established a chronic UVB-induced photoageing protocol in SKH-1 hairless mice. As shown in Fig. 3a, mice exposed to a 1·8 J cm^{-2} dose of UVB radiation displayed physically visible alternations, such as skin redness and burn spots, although this effect was reduced in mice treated topically with NS8-FS prior to UVB irradiation. Moreover, with chronic UVB irradiation, the thickened epidermal was observed in the histological stains, whereas the epidermal thickness was much lower in mice subjected to topical NS8-FS treatment (Fig. 3a). Quantitative epidermal thickness was measured by applying a digital scanner to stained skin samples (Fig. 3b).

We then used a non-invasive multiprobe adapter system to examine the effects of NS8-FS treatment on UVB-induced skin alterations. As shown in Fig. 3b, TEWL, which importantly reflects the skin barrier function, was elevated by the UVB radiation and was significantly lower in NS8-FS-treated skin, suggesting that topical NS8-FS treatment suppressed the UVB-induced decrease in the skin barrier function. In addition, exposure to UVB radiation induced a significant decrease in skin hydration compared with the control group, while the topical application of NS8-FS before UVB irradiation significantly retained skin moisture (Fig. 3b). The increased erythema value, due to the engorgement of vessels with oxygenated blood during exposure to UVB irradiation, was also reduced by NS8-FS protective treatment (Fig. 3b). No difference in the skin pH was observed among the three groups (data not shown). These results suggest the protective effectiveness of NS8-FS against UVB-induced skin photodamage.

Next, we investigated the regulation of NS8-FS on various key antioxidant enzymes, which play a central role in the impairment of the cellular defence system against ROS, such as CAT, SOD and GPX, from the skin of UVB-irradiated hairless mice. As shown in Fig. 3c, NS8-FS prevented the UVB-induced decrease in these antioxidant enzymes. Both the enzymatic activities of CAT and SOD were significantly different between the UVB-irradiated group and the NS8-FS-treated group, although the *P* value for the GPX levels between these two groups was 0·06. Moreover, enzyme activities measured in untreated and NS8-FS-treated mice were correlated with their expression using quantitative real-time PCR (supporting information Figure S2). Furthermore, we tested the levels of lipid peroxides, the hallmarks of photo-oxidative damage, in the skin of SKH-1 mice by measuring the lipid peroxidation product MDA. We found that the topical administration of NS8-FS could reduce the elevated lipid peroxidation of mice skin in response to UVB exposure (Fig. 3c). Therefore, it seems evident that the NS8-FS-induced protective effectiveness against UVB irradiation involves modulation of the skin antioxidant defence.

NS8-FS inhibited UVB irradiation-induced pigmentation in brown guinea pigs

In our study, we used guinea pig model to mimic human pigmentation. Three of four separate areas on the back of each guinea pig were topically treated with NS8-FS or 5% Kojic acid ahead of UVB irradiation or UVB alone. The UVB-induced skin pigmentation of the brown guinea pigs was visibly alleviated in the areas that received the NS8-FS topical treatment compared with the UVB-irradiated areas (Fig. 4a). Figure 4(b) presents the changes of the melanin index of the pigmented skin at the end point of the experimental period. Compared with the group treated with UVB alone, the melanin index of the skin was reduced by NS8-FS topical treatment, but the differences were not statistically significant for treatment with Kojic acid.

By using Fontana-Masson's staining on the skin samples, the granular melanins in the UVB groups were found to be greatly increased compared with the control group and were distributed throughout the basal layer. However, the melanin appearance in the NS8-FS groups was distinguishably reduced (Fig. 4c). Similarly, the number of TYR-positive melanocytes was increased and clustered after UVB irradiation, whereas TYR expression in the NS8-FS group was distinguishably reduced (Fig. 4c).

The protective effects of NS8-FS in the skin benefit from the upregulation of Nrf2

Nrf2 is a key transcriptional factor for antioxidant defence that can rapidly accumulate in the nucleus, in response to protective stimulation, and transactivate the expression of multiple antioxidant and phase II enzymes (Jung and Kwak 2010). Thus, we investigated the potential role of Nrf2 in the antioxidant response to NS8-FS, to protect skin cells against UVB-induced oxidative damage. First, we measured the Nrf2 expression in hairless mice groups at the transcriptional level, which increased approximately threefold after treatment with NS8-FS compared with UVB treatment alone (supporting information Figure S2). Then, in the *in vitro* setup, Nrf2

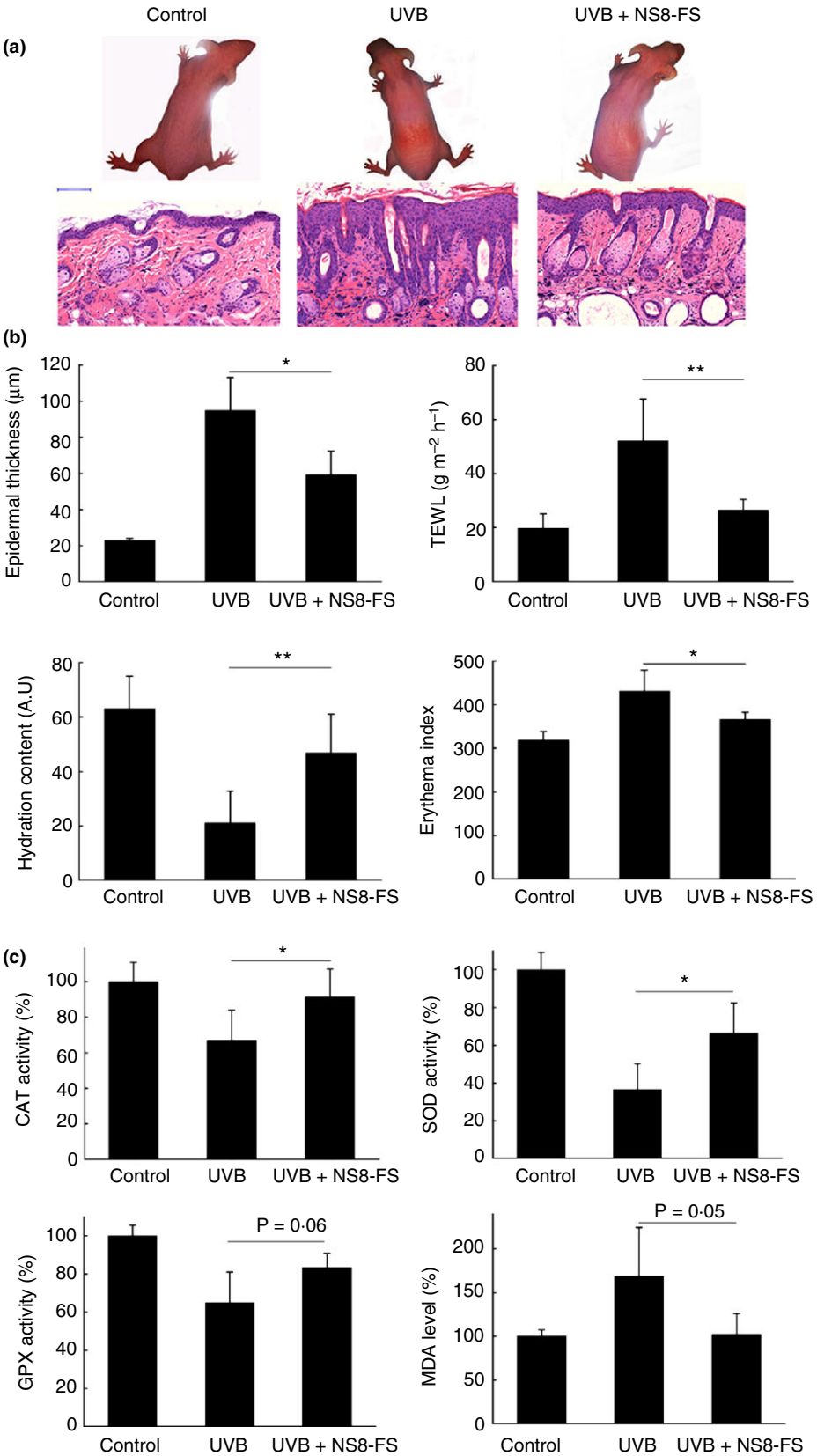
(a)
Control
UVB
UVB + NS8-FS
(b)
Epidermal thickness (μm)
TEWL (g m−2 h−1)
Hydration content (A.U)
Erythema index
(c)
CAT activity (%)
SOD activity (%)
GPX activity (%)
MDA level (%)
P = 0·06
P = 0·05

Figure 3 The protective effects of NS8-FS on the skin of mice receiving chronic UVB radiation. SKH-1 mice received continuous UVB radiation for 8 weeks with a total dose of 1·8 J cm^{-2}, $n = 10$ per group. Repetitive topical treatment with NS8-FS was applied before each UVB radiation. Representative pictures (a) showing UVB-induced dorsal sunburn and redness severity for the different groups indicated by haematoxylin-eosin staining of the skin samples (a). Epidermal thickness from at least four different positions of each slide was analysed with digital scanning software (b). After the radiation procedure, the main physiological skin parameters of mice were measured with a non-invasive Multiprobe Adapter System, including the TEWL, skin hydration and erythema (b). The catalase, superoxide dismutase and glutathione peroxidase activities (expressed as a % of control untreated mice) of skin samples obtained from SKH-1 mice were measured (c). The lipid peroxidation levels were also assayed by quantifying the end-product MDA (c). The results of (b, c) are expressed as the mean ± SEM, $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ comparing the NF8-FS-treated mice *vs* the group treated with UVB only. [Colour figure can be viewed at wileyonlinelibrary.com]

translocation into the nucleus increased as the concentration of added NS8-FS increased in HaCat cells (Fig. 5a). The results from the Western blot assays were confirmed by Nrf2 immunostaining, which demonstrated increased nuclear Nrf2-linked fluorescence in the presence of NS8-FS (Fig. 5b). These results suggest a close relationship between the photoprotective effect elicited by NS8-FS and its capacity to modulate the Nrf2-dependent antioxidant response.

Discussion

Based on the *in vitro* observations, we used two animal models (SKH-1 hairless mice and the brown guinea pig) to gain insight into whether *L. helveticus* NS8-fermented cell-free filtrate exerted protective effects on UV-induced skin photodamage. In this study, we focused on the skin antioxidant defence and the TYR related pathway and their response to topical NS8-FS. We found that NS8-FS exhibited comparable antioxidant and anti-melanogenesis activities both *in vitro* and *in vivo*, that the topical administration of NS8-FS effectively recovered the skin antioxidant defence through the activation of Nrf2 protein, and that the inhibition of the enzymatic activity and gene expression of TYR led to lighter pigmentation in the NS8-FS-treated group.

The antioxidant potential of LAB has been reported to originate from bacterial cells, the cellular lysate, released metabolites or hydrolysed milk components (Mishra *et al.* 2015). Still, distinguishing the compound or mechanism behind the antioxidant activity remains difficult. In our studies, the cell-free and casein-free supernatant of NS8-fermented milk exhibited more obvious radical scavenging and ROS inhibiting activities than that of NS8-fermented MRS culture medium (data not shown). Thus, we speculated that the antioxidant property of NS8-FS is likely caused by the proteolysed products released from *L. helveticus* NS8. Indeed, the development of radical scavenging activities during lactobacillus fermentation was connected to the produced peptides of low-molecular mass, depending on the specific proteolytic enzymes of the bacterial strains (Virtanen *et al.* 2007). Actually, bioactive peptides from fermented milk have attracted attention for their anti-hypertensive activity (Aleixandre *et al.* 2008). In addition, only a few antioxidant peptides have been identified in fermented dairy products (Qian *et al.* 2011; Sah *et al.* 2015). Investigations regarding the gene features of selected bacteria may expand our knowledge of these fermented metabolites with characterized capacities. Furthermore, as a major product of LAB fermentation, lactic acid has been used as a cosmetic ingredient for years and is thought to inhibit the enzymatic activity of TYR without influencing the expression of TYR (Usuki *et al.* 2003). In contrast, NS8-FS was proven to significantly suppress the expression of TYR and other key biosynthetic enzymes to melanosomes, so there appears to be another anti-melanogenesis component inhibiting the TYR pathway. Other studies have found that some short sequence peptides with TYR inhibitory activity can cause skin hypopigmentation (Ubeid and Hantash 2014). Taken together, further analysis of the specific component of NS8-fermented milk filtrate and additional data regarding the corresponding antioxidant or anti-melanogenesis activities are needed.

One important pathway that defends skin cells against oxidative stress is the phase II antioxidant response mediated through transcription factor Nrf2, which is maintained in an inactive form by binding to Keap-1. Under stimulation, the dissociated Nrf2 can translocate to the nucleus and bind to DNA as an antioxidant responsive element, and consequently initiates the transcription of antioxidant genes, such as hemeoxygenase-1, glutathione peroxidase, thioredoxin reductase, catalase and SOD (Jung and Kwak 2010; Gegotek and Skrzydlewska 2015). Upon UV light-mediated oxidative stress, Nrf2 silencing can promote melanogenesis in melanocytes, thereby modulating Nrf2 effects on the skin's tanning response (Chaiprasongsuk *et al.* 2015). Several natural compounds have been described to increase antioxidant capacities through the stimulation of the Nrf2 pathway in skin cells (Dinkova-Kostova *et al.* 2006; Soeur *et al.* 2015). We observed that pre-treatment with NS8-FS restored the UVB-mediated reduction of Nrf2 target antioxidant genes and proteins such as GPX, CAT and SOD and their corresponding enzymatic activities in the skin. Also, in cultured keratinocytes, NS8-FS stimulated Nrf2 stabilization

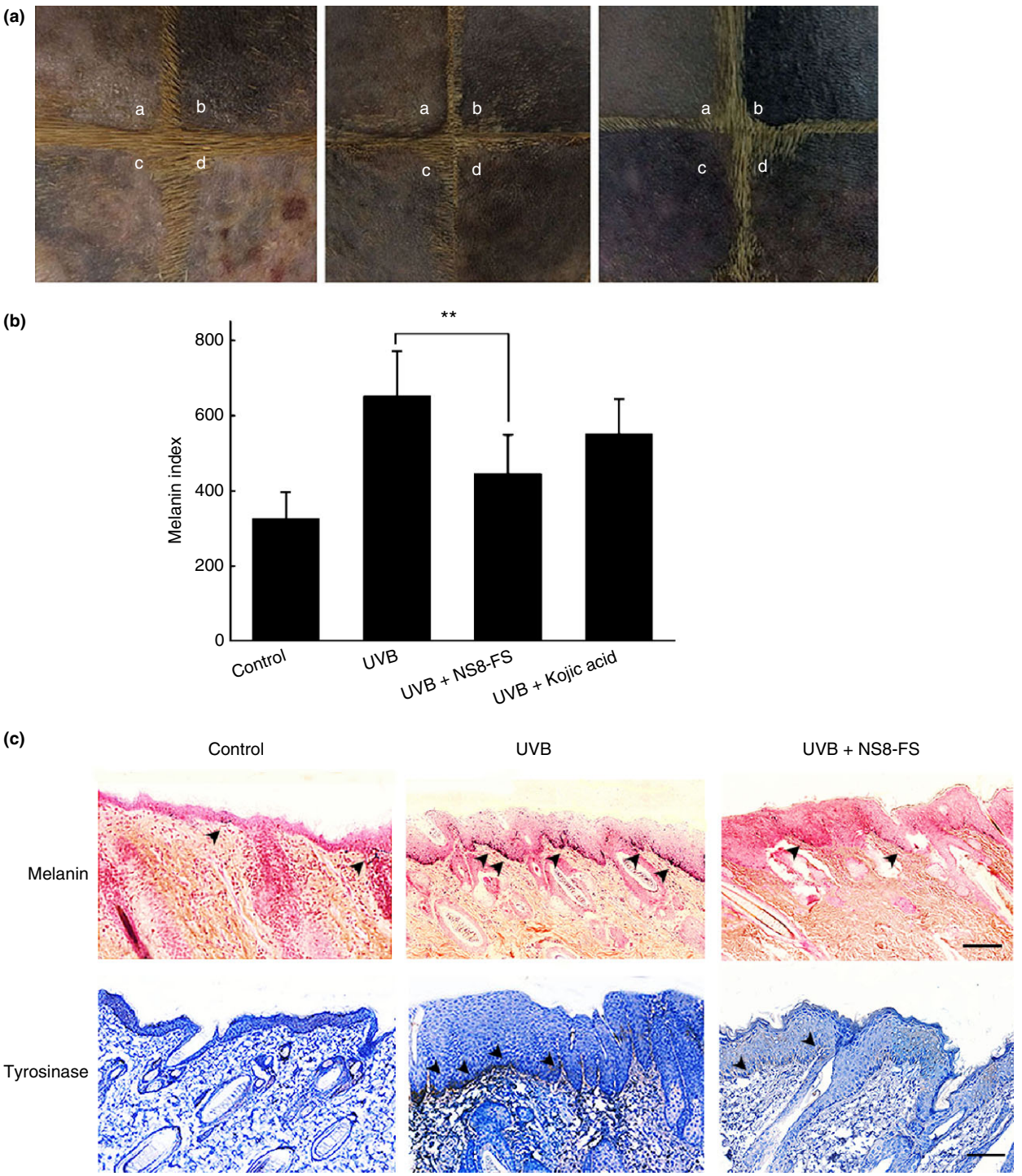

Figure 4 The effects of NS8-FS on pigmentation in UVB radiation-induced brown guinea pigs. The dorsal areas of the guinea pigs were shaved and received UVB radiation with an initial dose of 120 mJ cm^{-2} until distinguishable skin pigmentation appeared (n = 10 per group). The tanning colour of the guinea pigs was recorded with a digital camera (a). Skin from areas c and d was pre-treated with NS8-FS and Kojic acid (5%), respectively, before UVB radiation. Area a is the blank control without UVB radiation, and area b is the control area only treated with UVB. The melanin index was measured by the MPA5 probe (b). The melanin of skin samples was stained using the Masson-Fontana method (c). Skin tyrosinase levels were marked by IHC with anti-tyrosinase antibody (c). The data of (b) are expressed as the mean ± SEM. $^{**}P < 0{\cdot}01$ comparing NF8-FS-treated mice *vs* the group treated with UVB only. [Colour figure can be viewed at wileyonlinelibrary.com]

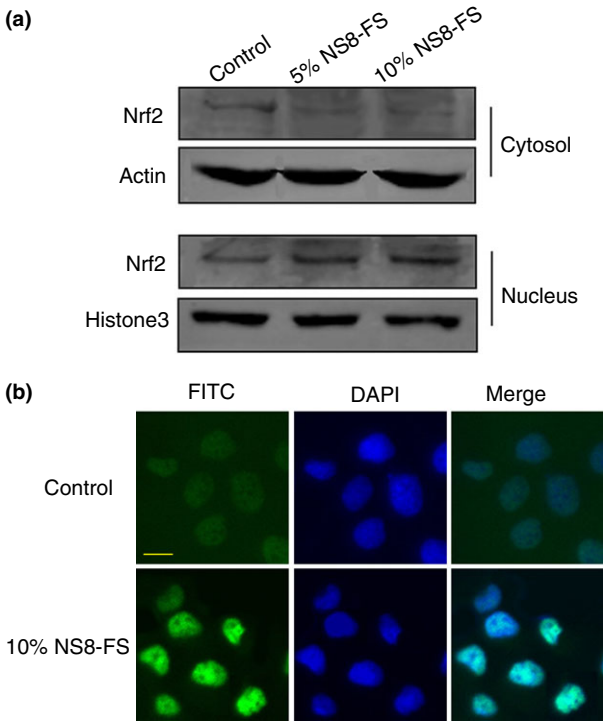

Figure 5 NS8-FS promotes Nrf2 activation. Cultured HaCat cells were treated with NS8-FS for 24 h. The expression of Nrf2 was detected by Western blotting the cytoplasm and nuclei fraction separately (a). Cellular immunostaining with anti-Nrf2 antibody was also performed to detect the activation of Nrf2 (b). [Colour figure can be viewed at wileyonlinelibrary.com]

and nuclear accumulation. Which component of NS8-FS acted as a potent Nrf2 inducer remains unknown. Present knowledge of probiotic strains and their influence on the Nrf2 signalling pathways is rather limited. *Lactobacillus plantarum* was found to confer antioxidant protective effects to hepatocytes through the activation of Nrf2 (Gao *et al.* 2013). In addition, some lactobacillus that originated from food were recently considered an important source of active compounds, which strongly supports the activation of the Nrf2 pathway in various cell types (Senger *et al.* 2016). To facilitate the application of LAB-fermented products as rational interventions for skin disorders, more studies should be carried out to define the mechanisms governing protective outputs against photooxidative damage and hyperpigmentation.

For models of UV-induced skin photodamage, several of the few available studies have administered the LAB or fermented milk orally, and elevated systematic protective mechanisms possibly explain the observed beneficial effects (Ra *et al.* 2014). However, topical treatment is the favoured form of skin care because it renders itself to local transdermal absorption and avoids systemic adverse effects. Thus, we first propose the topical application of lactobacillus-fermented milk supernatant as a skin care treatment. Our study presents the conclusive effects of NS8-FS discussed above and contributes information for evaluating the preventive or therapeutic functions of LAB-fermented products for skin care, which are ideal viable agents with applications in the cosmetics and pharmaceutical industries. Still, there are some limitations to our study. Because of the lack of quantitative measurements of NS8-FS due to the mixture compound of the fermented product, we completed the same set of *in vitro* or *in vivo* tests using the same batch of fermented product. Thus, we were unable to define the precise dose of treatment required to achieve a protective response. Although we did not observe any visible side effects from the topical use of NS8-FS in the models, its safety and stability require further evaluation. In future studies, we should focus on the component of NS8-fermented milk that effectively penetrates and deposits in the skin and its interaction with the ultimate target.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Inhibitory effects of NS8-FS on the gene expression of Tyrosinase and related proteins in B16F10 cells.

Figure S2. NS8-FS upregulates the expression of antioxidant enzymes and transcription factor Nrf2.