Cytoprotective effect of *Streptococcus thermophilus* against oxidative stress mediated by a novel peroxidase (EfeB)

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ABSTRACT

Streptococcus thermophilus is one of the most important starter species used in the dairy industry and exhibits several beneficial properties for the hosts. However, knowledge of the mechanism of its beneficial effect is still limited. The objective of this study was to investigate the cytoprotective effect of *S. thermophilus* CGMCC 7.179 with a novel peroxidase (EfeB) against oxidative stress in human intestinal epithelial cells, HT-29. Previously, we identified EfeB in *S. thermophilus* CGMCC 7.179, which could provide protection when growing at aerobic conditions. Here, we found that, when exposed to 15 mM H₂O₂, the cell viability of the *efeB* mutant (ST1314) was much lower than that of strain CGMCC 7.179, and the 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity of strain ST1314 decreased by 15%. When co-incubated with HT-29 cells, strain CGMCC 7.179 stimulated the enhancement of the major antioxidant enzyme activities (superoxide dismutase, glutathione peroxidase, and catalase) in HT-29 cells under 2 mM H₂O₂-induced oxidative stress, whereas the active decrease of those antioxidant enzymes was observed in strain ST1314. In addition, the intracellular reactive oxygen species content in HT-29 cells co-incubated with strain CGMCC 7.179 was lower than that with strain ST1314 under the same oxidative stress. Furthermore, the protein content of nuclear factor erythroid 2-related factor 2 (Nrf2) in HT-29 cells following strain CGMCC 7.179 treatment was 1.4-fold higher than that with strain ST1314 treatment, and the increased transcription levels of Nrf2-related antioxidant enzyme genes were also observed in strain CGMCC 7.179 cells. All of these results demonstrated that *S. thermophilus* CGMCC 7.179 enhanced cellular antioxidant responses and endowed host cells with pro-

TECTIVE effects against oxidative stress mediated by the peroxidase EfeB.

Key words: *Streptococcus thermophilus*, peroxidase EfeB, oxidative stress, intestinal epithelial cell

INTRODUCTION

The occurrence of oxidative stress is due to an imbalance between production of reactive oxygen species (ROS) and antioxidant capabilities of cellular antioxidants in a biological system (Halliwell, 2009). Although oxidation is essential to many living organisms for the production of energy to promote biological processes, excessive ROS and oxidation products cause oxidative damage to cellular biomolecules such as proteins, nucleic acids, and lipids, impairing their biological functions (Berlett and Stadtman, 1997). Thus, oxidative stress is related to the pathogenesis of multiple chronic health problems and diseases, including inflammation, age-related diseases, and cancers (Storz, 2005; Valko et al., 2007; Spooner and Yilmaz, 2011). Substantial evidence has indicated that generation of an enhanced level of ROS leads to the development of some chronic intestinal inflammatory diseases and is commonly implicated in the pathogenesis of inflammatory bowel diseases, which have been recognized as chronic inflammatory disorders of the human gastrointestinal tract (Kruidenier and Verspaget, 2002; Zhu and Li, 2012). For this, the human body has evolved various cellular defense mechanisms to protect organisms from ROS overproduction and oxidative stress. The key antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-px), and catalase (CAT) are able to trigger these mechanisms against oxidative burst and offer protection to cells and tissues against oxidative injury (Bonnefont-Rousselot et al., 2000). Furthermore, nuclear factor erythroid 2-related factor 2 (Nrf2), known as a major regulator of a cellular defense system against oxidative stress, is able to control the antioxidant response element (ARE)-dependent gene regulation to respond to oxidative stress in human cells (Jaiswal, 2004).

It has been claimed repeatedly that some microorganisms, particularly the species of lactic acid bacteria

Received February 16, 2018.

Accepted April 18, 2018.

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(LAB), which are widely used in the manufacture of fermented products, possess antioxidant properties, and confer a health benefit for the hosts (Pagnini et al., 2010; Martarelli et al., 2011). Considered a generally recognized as safe (GRAS) species (Hols et al., 2005), *Streptococcus thermophilus* is the most frequently used species in the fermentation of yogurt and cheeses as starter cultures in the dairy industry (Morelli L, 2014). The recovery of *S. thermophilus* in feces of human volunteers consuming yogurt has definitively established that this bacterium remains alive during its transit through the digestive tract (Mater et al., 2005; Elli et al., 2006). Furthermore, it has been reported that *S. thermophilus* in yogurt cultures could promote lactose digestion in lactose-intolerant patients and also stimulate the gut immune system (Rabot et al., 2010; Savaiano, 2014). Although *S. thermophilus* exhibits several beneficial properties for the host, only a few studies have been focused on the mechanism of its beneficial effects.

Streptococcus thermophilus has evolved a specific inducible defensive system against ROS damage to make itself grow in the presence of oxygen and survive in the presence of a low concentration of H_2O_2 (Thibessard et al., 2001b). Those defensive systems were in conjunction with the NADH oxidase, SOD, glutathione, thioredoxin, and other antioxidant systems in *S. thermophilus* (Chang and Hassan, 1997; Hols et al., 2005; Marco et al., 2013; Lu and Holmgren, 2014; Wang et al., 2017). Previously, we identified a novel dye-decolorizing peroxidase EfeB, the only peroxidase found in *S. thermophilus*, which played a role in protecting cells of *S. thermophilus* from oxidative stress, with the assistance of the Tat system (Zhang et al., 2015). The antioxidant effect of LAB is one of the most important mechanism for health benefit of the host; thus, in this study, we aimed to investigate the cytoprotective effect of *S. thermophilus* GCMCC 7.179 against oxidative stress in human intestinal epithelial cells, and the mechanism mediated by EfeB.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Streptococcus thermophilus CGMCC7.179, originally isolated from a traditional yogurt of Inner Mongolia, China, and its *efeB* isogenic mutant ST1314 (Zhang et al., 2015) were used in this study. *Streptococcus thermophilus* strains were propagated in M17 broth (Oxoid, Basingstoke, UK) supplemented with 1% (wt/vol) of lactose (LM17 broth) at 42°C without shaking. All cultures were grown for 12 h (stationary phase) before being used in the experiments.

Detection of Cell Survival Against Oxidative Stress

Cell survival under H_2O_2 treatment was detected as described previously (Thibessard et al., 2001a). Briefly, after overnight static incubation at 42°C, *S. thermophilus* CGMCC7.179 and ST1314 were 2% inoculated into 5 mL of fresh LM17 broth and incubated statically at 42°C. When the optical density at 600 nm reached 0.4, cell cultures were treated with 15 mM H_2O_2 for 30 min. The survival of *S. thermophilus* cells against oxidative stress was calculated by pipetting samples onto plates and determining the numbers of colony-forming units according to a previous method (Zhang et al., 2015).

Scavenging Activity of 1,1-Diphenyl-2-Picrylhydrazyl Radical

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of cell-free extract was analyzed as described previously with minor modifications (Lin and Chang, 2000). Cell-free extracts were obtained from cell suspensions by shaking in a bullet blender of 0.1 g of glass beads for 30 s, 3 times, and centrifuged at $13,000 \times g$ for 10 min at 4°C to remove the cell debris. The protein concentration of cell-free extracts was determined by UV-Vis Spectrophotometer Q5000 at a wavelength of 280 nm (Quawell, Sunnyvale, CA). Sodium phosphate buffer (0.2 M Na_2HPO_4 , 0.2 M NaH_2PO_4 , 2 mM EDTA, pH 7.4) was used in this assay.

Then, a sample of 200 μ L of cell-free extract or buffer as reference was treated with 200 μ L of freshly prepared DPPH solution (0.2 mM in alcohol) in the absence of light. After incubation of 30 min, the reaction mixture was centrifuged at $13,000 \times g$ for 10 min at room temperature, and the suspension was obtained. The scavenged DPPH was then monitored by measuring the reduction in absorbance at 517 nm. The DPPH scavenging ability was defined as follows: $[1 - A_{517}(\text{sample})/A_{517}(\text{reference})] \times 100$.

Epithelial Cell Lines and Culture Conditions

The HT-29 human colonic epithelial cell line was used in this study. The cells were grown in RPMI 1640 medium (Hyclone, Logan, UT) containing 10% (vol/vol) fetal calf serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), 1% (vol/vol) penicillin-streptomycin solution (100 U/mL of penicillin and 100 μ g/mL of streptomycin, Hyclone) at 37°C in a humidified atmosphere in the presence of 5% CO_2 . After reaching 80% confluence, the cells were detached using 0.25% trypsin-EDTA (Hyclone) and subsequently seeded in 6-well culture plates at a density of 8×10^5 cells/well for the antioxidant assays. After 24 h growth, cells were

washed twice with PBS (Hyclone) and then co-incubated with 1×10^8 cfu/mL of *S. thermophilus* strains (optical density at 600 nm of 0.6–0.8) in FBS-free medium for 2 h under the conditions described above for the following experiments. Cells in fresh medium without FBS were regarded as control.

Measurement of Intracellular SOD, GSH-px, and CAT

For these assays, *S. thermophilus*-treated cells were washed twice and then incubated in 2 mL of fresh medium without FBS containing 2 mM H_2O_2 for 30 min to induce oxidative stress. After the treatment, the cells were washed twice and lysed using cell lysis buffer with 1 mM phenylmethanesulfonyl fluoride (Beyotime Biotechnology, Shanghai, China). The lysates were collected by scraping from the plates and centrifuged at $13,000 \times g$ at 4°C for 5 min. The supernatants were used for further determination of SOD, GSH-px, and CAT immediately. The activities of SOD, GSH-px, and CAT level were measured using the corresponding activity assay kits (Nanjing Jiancheng Bioengineering Corporation, Nanjing, China) according to the commercial instructions (<http://www.njcbio.com/uploadfile/product/big/20180503153958447.pdf>). Protein concentration in the supernatant was determined by the UV-Vis Spectrophotometer at Q5000 at a wavelength of 280 nm.

Measurement of Intracellular ROS

The intracellular ROS was detected according to a method described previously with minor modifications (Li and Shah, 2016). Briefly, after co-incubation with *S. thermophilus* strains, the oxidative burst was induced by H_2O_2 at a final concentration of 2 mM for 30 min and quantified using flow cytometry as a measure of ROS production following H_2O_2 activation. The assay depends upon the cell incorporating DCFH-DA at a final concentration of 10 μM as a stable nonfluorescent probe. The 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was cleaved in DCFH by cellular esterase and then oxidized to the fluorescent dichlorofluorescein under the action of ROS. The cells were then harvested by trypsin and washed with PBS to remove excess DCFH-DA. Then, the fluorescence data of 10,000 cells were recorded for flow cytometry (ImageStreamX Mark II, Amnis, Seattle, WA). Results were expressed as mean fluorescence intensity.

Immunoblotting for Nrf2 Expression

After co-incubation with bacterial strains, the cells were washed twice and lysed by the methods described

above. Equal amounts of cellular protein samples (typically 30 ng) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Solarbio, Beijing, China) for 2 h. After being blocked with Tris-buffered saline with polysorbate 20 containing 5% nonfat dried milk (Becton, Dickinson and Co., Sparks, MD) for 1 h at room temperature, the membranes were incubated overnight at 4°C with a rabbit polyclonal antibody against Nrf2 and β -actin as reference (1:1,000 dilution) and subsequently probed with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000 dilution) for 1 h at room temperature. The bands were visualized by enhanced chemiluminescence agents.

Analysis of The Gene Transcriptions by Real-Time PCR

After co-incubation with bacterial strains, the total RNA of the cells was extracted using an RNA Simple total RNAprep Pure Cell/Bacteria kit (Tiangen, Beijing, China) according to the manufacturer's protocols (<http://www.tiangen.com/asset/imsupload/up0015629001467351068.pdf>). The appropriate mass of the total RNA was subjected to perform reverse transcription to synthesize cDNA, using a PrimeScript RT reagent kit (Takara, Shiga, Japan) according to the manufacturer's instructions (<http://www.takarabiomed.cn/Download/RR047A.pdf>). To quantify the gene transcription level, real-time quantitative PCR was carried out with SYBR Premix Ex TaqII (Takara) using a thermal cycler (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocols. The primer pairs used for real-time quantitative PCR are shown in Table 1. Relative expression level for each gene was normalized against β -actin as an internal standard and calculated by the comparative threshold cycle (C_T) method ($2^{-\Delta\Delta C_T}$).

Statistical Analysis

Experimental data were reported as the mean $\pm$ standard deviation. Statistical significance between treatment and control conditions was assessed by 1-way ANOVA and unpaired 2-tailed Student's *t*-tests. Probability values <0.05 were considered statistically significant.

RESULTS

Characterization of EfeB Against Oxidative Stress

To investigate the effect of EfeB on defending oxidative stress, the *efeB*-deficient mutant ST1314 was

Figure 1. Cell survival of *Streptococcus thermophilus* CGMCC 7.179 (WT) and ST1314 (Δ *efeB*) under normal growth conditions (gray bar) and oxidative stress induced by 15 mM H_2O_2 (white bar) was determined in M17 broth (Oxoid, Basingstoke, United Kingdom) supplemented with 1% (wt/vol) of lactose (LM17 broth). Data are shown as the mean $\pm$ SD, $n = 3$. Asterisks represent a statistically significant difference compared with WT under oxidative stress (* $P < 0.05$).

Figure 2. The scavenging activity of *Streptococcus thermophilus* CGMCC 7.179 (WT) and ST1314 (Δ *efeB*) on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was detected. Values are expressed as mean $\pm$ SD, $n = 3$. *Significantly lower than WT strain at $P < 0.05$.

Antioxidant Enzyme Activities in *S. thermophilus*-Treated HT-29 Cells

constructed in *S. thermophilus* CGMCC 7.179 by homologous double-crossover recombination (Zhang et al., 2015). Zhang et al. (2015) showed that strain ST1314 resulted in decreased growth of the strain CGMCC 7.179 at aerobic conditions. To further confirm the protective effect of EfeB against oxidative stress, the cell viabilities of *S. thermophilus* CGMCC 7.179 and ST1314 were determined under 15 mM H_2O_2 -induced oxidative stress. The results showed no change between the 2 strains without oxidative stress, whereas the cell viability of strain ST1314 was 2 orders of magnitude lower than strain CGMCC 7.179 under H_2O_2 -induced oxidative stress (Figure 1).

To further investigate the antioxidant properties of *S. thermophilus* CGMCC 7.179 conferred by EfeB, the capabilities of DPPH radical scavenging of strain CGMCC 7.179 and ST1314 were determined. The results showed that DPPH scavenging activities of strain ST1314 declined from 50% of strain CGMCC 7.179 to 43% (Figure 2).

To investigate the effect of *S. thermophilus* CGMCC 7.179 on antioxidant activity in intestinal epithelial cells, the intracellular activities of 3 antioxidant enzymes in HT-29 cells were evaluated under 2 mM H_2O_2 -induced oxidative stress. First, the concentration of H_2O_2 used was determined by preliminary experiments: concentrations of H_2O_2 ranging from 10 μ M to 5 mM were analyzed for the HT-29 cell line, and 2 mM induced maximal oxidative burst, whereas cell viability was maintained at 70% (data not shown). The effect of strain CGMCC 7.179 on SOD activity in HT-29 cells is shown in Figure 3A. When exposed to H_2O_2 alone, the activity of SOD sharply decreased from 7.55 U/mg of protein in the normal cells to 4.69 U/mg of protein. When co-incubated with strain CGMCC 7.179, SOD activity reached 8.19 U/mg of protein, a 1.7-fold increase compared with those of H_2O_2 -treated cells, whereas SOD activity of strain ST1314 treatment was 5.67 U/mg of protein, 30% lower than strain CGMCC 7.179. The *tert*-butylhydroquinone (100 μ M), a syn-

Table 1. Real-time quantitative PCR primers used for quantification of human mRNA

Gene name	Forward (from 5' to 3')	Reverse (from 5' to 3')
<i>Nrf2</i>	ACACGGTCCACAGCTCATC	TGTCAATCAAATCCATGTCCTG
<i>HO1</i>	CTTCTTCACCTTCCCCAACA	AGCTCCTGCAACTCCTCAAA
<i>GCLC</i>	GGAGACCAGAGTATGGGAGTT	CCGGCGTTTTTCGCATGTTG
<i>NQO1</i>	ATGTATGACAAAGGACCCTTCC	TCCCTTGCAGAGAGTACATTGG
<i>TXNRD1</i>	ATATGGCAAGAAGGTGATGGTCC	GGGCTTGTCCTAACAAAGCTG
<i>ACTB</i>	AGTTGCGTTACACCCTTTCTTG	TCACCTTCACCGTTCCAGTTT

thetic antioxidant, was used as a positive control in these experiments (Abiko et al., 2011).

To further confirm the effect of *S. thermophilus* CGMCC 7.179 on antioxidant enzyme activity in HT-29 cells, the GSH-px activity was determined in strain CGMCC 7.179 and ST1314-treated HT-29 cells under 2 mM H₂O₂, respectively. As shown in Figure 3B, the intracellular GSH-px level in H₂O₂-treated cells decreased about 2-fold compared with the normal cells, whereas the GSH-px activity in strain CGMCC 7.179-treated cells was noticeably enhanced 1.5-fold compared with H₂O₂-treated cells. However, strain ST1314-treated cells failed to increase the level of GSH-px.

In addition, CAT activity was assayed. Treatment of 2 mM H₂O₂ caused a prominent decline in CAT activity in HT-29 cells, whereas co-incubation with *S. thermophilus* CGMCC 7.179 significantly enhanced the CAT activity 3-fold compared with the H₂O₂ group, and the CAT activity of strain ST1314 treatment was not increased (Figure 3C).

Determination of Intracellular ROS Production

To assess the capability of *S. thermophilus* CGMCC 7.179 to reduce H₂O₂-induced intracellular ROS generation, the level of ROS formation in HT-29 cells was monitored using the dichlorofluorescein fluorescence probe, which was directly proportional to ROS content. The oxidative burst was induced by H₂O₂ at a concentration of 2 mM. As shown in Figure 4, when the cells were exposed to 2 mM H₂O₂ alone, the intracellular ROS level was increased 2-fold compared with the endogenous ROS production, and a 20% reduction of the ROS amounts was observed in HT-29 cells co-incubated with strain CGMCC 7.179 compared with H₂O₂ alone, whereas this reduction of ROS was not obvious in strain ST1314-treated cells. These results indicated that *S. thermophilus* CGMCC 7.179 could attenuate H₂O₂-induced ROS production in the presence of EfeB.

Effect of *S. thermophilus* CGMCC 7.179 on Cellular Expression of Nrf2 Protein

To clarify whether the antioxidant effect of *S. thermophilus* CGMCC 7.179 for the host cells was due to activation of the Nrf2, a master regulator in the cellular antioxidant systems, Nrf2 protein level was determined by immunoblotting. The results showed that co-incubation with strain CGMCC 7.179 promoted Nrf2 protein level 1.4-fold compared with the normal group in HT-29 cells, whereas no obvious change was observed in Nrf2 protein level of strain ST1314-treated HT-29 cells (Figure 5A).

Figure 3. Effects of *Streptococcus thermophilus* CGMCC 7.179 on superoxide dismutase (SOD) activity (A), glutathione peroxidase (GSH-px) activity (B), and catalase (CAT) activity (C) in HT-29 cells. The HT-29 cells were co-incubated with positive control [*tert*-butylhydroquinone (tBHQ)], strain CGMCC 7.179 (WT), and ST1314 (Δ eFeB) in the presence of H₂O₂. Values are expressed as mean $\pm$ SD, n = 3. The normal group represented the cells with no treatment. The control group represented the cells incubated with H₂O₂ alone. Asterisks represent a statistically significant difference compared with the control group (**P* < 0.05; ***P* < 0.01). prot = protein.

Figure 4. Effects of *Streptococcus thermophilus* CGMCC 7.179 on H_2O_2 -induced reactive oxygen species production in HT-29 cells by flow cytometry analysis. The HT-29 cells were co-incubated with positive control [*tert*-butylhydroquinone (tBHQ)], strain CGMCC 7.179 (WT), and ST1314 ($\Delta efeB$), in the presence of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) and H_2O_2 . The normal group used cells incubated with DCFH-DA alone (no stress induction); the control group used cells incubated with DCFH-DA and H_2O_2 (stress induction). Values are expressed as mean $\pm$ SD, $n = 3$. Asterisks represent a statistically significant difference compared with the control group (* $P < 0.05$; ** $P < 0.01$).

Effect of *S. thermophilus* CGMCC 7.179 on Gene Expression of Antioxidant Enzymes

To further verify the antioxidant effect of *S. thermophilus* CGMCC 7.179 mediated by Nrf2, the gene expressions of several antioxidant enzymes regulated by Nrf2 were evaluated in HT-29 cells. The results first showed that the transcription level of *Nrf2* gene in HT-29 cells co-incubated with strain CGMCC 7.179 was 2-fold higher than normal group, whereas strain ST1314 treatment did not show this enhancement (Figure 5B). Additionally, following strain CGMCC 7.179 treatment, the mRNA expressions of other antioxidant enzymes including *HO1*, *GCLC*, *NQO1*, and *TXNRD1* were significantly increased, and not promoted obviously in strain ST1314-treated HT-29 cells as we hypothesized (Figure 6).

DISCUSSION

Accumulated evidence revealed that LAB exerts multiple biological roles through several mechanisms, the antioxidant activity being one of the most debated (Rossi and Amaretti, 2010). In fact, among various beneficial effects for LAB, the cytoprotective effect against oxidative stress was focused on in previous

Figure 5. Effect of *Streptococcus thermophilus* CGMCC 7.179 on the cellular nuclear factor erythroid 2-related factor 2 (Nrf2) protein level as determined by immunoblot analysis (A) and transcription level of the *Nrf2* gene as determined by real-time quantitative PCR (B). The HT-29 cells were co-incubated with strain CGMCC 7.179 (WT) and ST1314 ($\Delta efeB$) for 2 h. Relative protein content and gene transcription level were presented as the fold change relative to the normal group, respectively. The β -actin was used as an internal reference. Data are shown as the mean $\pm$ SD, $n = 3$. Asterisks represent a statistically significant difference compared with the normal group (* $P < 0.05$; ** $P < 0.01$).

studies (Songisepp et al., 2005; Virtanen et al., 2007). In this study, we found that *S. thermophilus* CGMCC 7.179 with EfeB activity, which endowed the strain with antioxidant effects, could stimulate the activities of antioxidant enzymes (SOD, GSH-px, and CAT) in human intestinal epithelial cells HT-29, and effectively attenuate intracellular ROS overproduction. Furthermore, in the presence of EfeB, the protein level of Nrf2 and the transcription level of Nrf2-related antioxidant

Figure 6. Effects of *Streptococcus thermophilus* CGMCC 7.179 on gene expression of antioxidant enzymes. The gene transcription levels of the antioxidant enzymes *HO1*, *GCLC*, *NQO1*, and *TXNRD1* were evaluated after co-incubation with strain CGMCC 7.179 (WT) and ST1314 (Δ efeB) for 2 h. Relative gene expression levels were presented as the fold change relative to the normal group. Data are shown as the mean $\pm$ SD, $n = 3$. Asterisks represent a statistically significant difference compared with the normal group (* $P < 0.05$; ** $P < 0.01$).

enzyme genes were significantly increased in HT-29 cells co-incubated with *S. thermophilus* CGMCC 7.179, suggesting that *S. thermophilus* CGMCC 7.179 with EfeB was able to accomplish the function of antioxidant effects for host cells by controlling the Nrf2 pathway.

As the second most important starter species used in dairy industry after *Lactococcus lactis*, about 10^{21} live cells of *S. thermophilus* were ingested by humans in a previous study (Hols et al., 2005). Therefore, except for the excellent character of fermentation and flavor formation, *S. thermophilus* has several probiotic properties in human gastrointestinal cells. Li and Shah (2017) reported that EPS with sulfonated modification from *S. thermophilus* was effective in improving antioxidant activities. Based on these, we set out to investigate the mechanism of antioxidant activity of *S. thermophilus*

for human intestinal epithelial cells. It has been reported that LAB-producing antioxidant enzymes such as SOD and CAT can enhance the cellular antioxidant and anti-inflammatory activity (Bruno-Barcena et al., 2004; Del Carmen et al., 2014), suggesting that the antioxidant effect of LAB may be related to the expression of antioxidant enzymes. Our previous study has identified a novel dye-decolorizing peroxidase EfeB in *S. thermophilus* CGMCC 7.179, which could provide protection when grown at aerobic conditions. The H_2O_2 stress assay in our study further confirmed that this enzyme played a protective role against oxidative stress in *S. thermophilus* CGMCC 7.179. The DPPH assay has been widely used to estimate the free radical scavenging effectiveness of various antioxidant substances, and it is regarded as an indicator of antioxidant activity evalu-

ation for LAB. That DPPH scavenging activities of strain ST1314 were lower than that of strain CGMCC 7.179, suggesting that EfeB plays an important role in antioxidant activity of *S. thermophilus* CGMCC 7.179.

Oxidative stress mainly presents as the overproduction of intracellular ROS. It has been reported that a SOD-producing recombinant *Lactobacillus casei* BL23 strain could attenuate ROS generation, demonstrating the antioxidant effect of *Lb. casei* expressing MnSOD in cellular models under oxidative stress (Watterlot et al., 2010). Our result also proved that in the presence of EfeB, *S. thermophilus* CGMCC 7.179 could relieve ROS production in intestinal epithelial cells. In addition, SOD, GSH-px, and CAT are known to be the 3 primary antioxidant enzymes in all oxygen metabolizing cells to coordinate intracellular redox homeostasis and eliminate excessive ROS. Thus, the activities of SOD, GSH-px, and CAT were determined to evaluate the changes of antioxidant status in the cells. Our results showed that *S. thermophilus* CGMCC 7.179 was capable of enhancing the activities of antioxidant enzymes in human intestinal epithelial cells, whereas strain ST1314 did not exhibit significant enhancement, indicating that *S. thermophilus* CGMCC 7.179 with EfeB activity had the capability to protect cells against oxidative damage via increasing SOD, GSH-px, and CAT activities, and it was able to regulate the balance of oxygenation effectively in the cells under oxidative stress.

The Nrf2 serves as master regulator of a cellular defense system against oxidative stress, controlling the expression of multiple antioxidant enzymes with an ARE in their promoter regions (Jaiswal, 2004). Under normal conditions, Nrf2 is suppressed by binding to the Kelch-like ECH-associated protein 1 and localized in the cytoplasm in an inactive form, followed by ubiquitin-dependent proteasomal degradation. Under oxidative stress, conformational changes caused by electrophilic xenobiotics or oxidation products in Kelch-like ECH-associated protein 1 lead to the release of Nrf2, thus removing it from degradation. Subsequently, Nrf2 translocates into the nucleus where it binds to the ARE of antioxidant and phase II detoxification enzyme genes to activate their expression and activity (Taguchi et al., 2011). The transcriptional activation of more than 200 antioxidant and protective genes may be controlled by Nrf2. As we know, the Nrf2-ARE pathway is responsible for stimulating SOD, GSH-px, and CAT expression, and hence constitutes a crucial cellular response to oxidative stress (Surh et al., 2009). Therefore, we speculated that the antioxidant effect of *S. thermophilus* CGMCC 7.179 mediated by EfeB was associated with the activation of Nrf2. As expected, the immunoblotting data clearly showed that the *S. thermophilus* CGMCC 7.179 treatment promoted the protein expres-

sion of Nrf2 in HT-29 cells, whereas strain ST1314 also failed to show an increase, suggesting that this strain was capable of activation of Nrf2 mediated by EfeB. Additionally, *S. thermophilus* CGMCC 7.179 with EfeB activity increased the transcription levels of antioxidant enzymes in HT-29 cells as well as enhanced Nrf2 protein level, further demonstrating that the strain conferred a higher antioxidant protection for host cells through the Nrf2 pathway by the function of EfeB.

CONCLUSIONS

To our knowledge, this is the first time that cytoprotective effect against oxidative stress has been achieved for *S. thermophilus* carrying a novel peroxidase, EfeB. Our findings suggest that *S. thermophilus* CGMCC 7.179 that express EfeB and that are able to modulate antioxidant responses by Nrf2 could be useful in the development of functional products for the food industry with beneficial effects.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Natural Science Foundation of China (31471715), National Key Research and Development Program of China (2017YFD0400300), and Public Service Sectors (Agriculture) Special and Scientific Research Projects (201503134; Beijing).

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