Appl Microbiol Biotechnol (2017) 101:3015–3026
DOI 10.1007/s00253-016-8032-4

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Bacillus amyloliquefaciens SC06 alleviates the oxidative stress of IPEC-1 via modulating Nrf2/Keap1 signaling pathway and decreasing ROS production

Yang Wang[1] · Yanping Wu[1] · Yibing Wang[1] · Aikun Fu[1] · Li Gong[1] · Weifen Li[2] · Yali Li[2]

Received: 19 June 2016 / Revised: 23 November 2016 / Accepted: 26 November 2016 / Published online: 12 December 2016

Abstract Oxidative stress (OS) plays a major role in the gastrointestinal disorders. Although probiotics were reported to repress OS, few researches compared the antioxidant ability of different *Bacillus* strains and deciphered the mechanisms. To select a *Bacillus* strain with higher antioxidant capacity, we used H_2O_2 to induce intestinal porcine epithelial cell 1 (IPEC-1) OS model. The most suitable H_2O_2 concentration and incubation time were determined by the half lethal dose and methyl thiazolyl tetrazolium. Correlation analysis was performed to choose a sensitive indicator for OS. As for the comparison of *Bacillus*, cells were divided into control, *Bacillus* treatment, H_2O_2 treatment, and *Bacillus* pre-protection + H_2O_2 treatment. *Bacillus* were co-cultured with IPEC-1 for 3 h in *Bacillus* and *Bacillus* pre-protection + H_2O_2 treatments. Then, based on OS model, 300 μmol/L H_2O_2 was added into medium of H_2O_2 and *Bacillus* pre-protection + H_2O_2 treatments for another 12 h. Antioxidant and apoptosis gene expressions were detected to screen the target strain. Nuclear factor erythroid-derived 2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein1 (Keap1) pathway, reactive oxygen species (ROS) production, mitochondrial membrane potential (Δψm), apoptosis, and necrosis were analyzed. Results revealed that heme oxygenase-1 (*HO-1*) gene expression had a positive correlation with H_2O_2 induction. Moreover, *Bacillus amyloliquefaciens* SC06 (SC06)-meditated IPEC-1 showed the best antioxidant capacity though modulating Nrf2 phosphorylation. Δψm was elevated, while ROS generation was reduced with SC06 pre-protection, resulting in decreased apoptosis and necrosis. Altogether, *HO-1* expression could be regarded as an OS indicator. The regulation of Nrf2/Keap1 pathway and ROS production by SC06 are involved in alleviating OS of IPEC-1.

Keywords *Bacillus* · IPEC-1 · Oxidative stress · Nrf2 · ROS · NOX

Introduction

Oxidative stress (OS) basically defines a condition in which prooxidant–antioxidant balance in the cell is disturbed, resulting in DNA hydroxylation, protein denaturation, lipid peroxidation, and apoptosis, ultimately compromising cells' viability (Sies 1997; Valko et al. 2006; Sinha et al. 2013). Since intestine is the biggest digestive organ, intestinal OS influences nutrition digestibility and absorption, resulting in damaged animal growth and development (Zhang et al. 2013). Intestinal epithelium, sitting at the interface between the organism and its luminal environment, is prone to OS induced by luminal oxidants (Circu and Aw 2012). OS is often associated with the accumulation of reactive oxygen species (ROS), such as hydroxyl radical and the superoxide radical. ROS has extremely high reactivity, which gradually leads to

Yang Wang and Yanping Wu contributed equally to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-016-8032-4) contains supplementary material, which is available to authorized users.

✉ Weifen Li
wfli@zju.edu.cn

✉ Yali Li
liyali06@163.com

[1] Key Laboratory of Molecular Animal Nutrition of the Ministry of Education, Institute of Feed Science, College of Animal Sciences, Zhejiang University, Hangzhou 310058, China

[2] Key Laboratory of Molecular Animal Nutrition of the Ministry of Education, Institute of Feed Science, College of Animal Sciences, Zhejiang University, Hangzhou 310058, China

oxidative damage to biomolecules. In cells, one of the major enzymatic sources of cellular ROS generation is nicotinamide adenine dinucleotide phosphate oxidase (NOX). Activation of NOX is involved in the recruitment of multiple cytosolic subunits including $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac with its membrane-bound subcomplex consisting of $gp91^{phox}$ and $p22^{phox}$ and resulting in the rapid generation of large amounts of superoxide anion (Park et al. 2003; Lambeth 2004).

The maintenance of the intestinal epithelial redox environment is essential for the activities of key physiological processes, which includes digestion and absorption, cell proliferation and apoptosis, and immune response (Circu and Aw 2012). To combat with OS, cells develop their own antioxidant machineries consisting of several antioxidant enzymes such as heme oxygenase-1 (HO-1), superoxide dismutase (SOD) enzyme family (SOD-1, SOD-2, SOD-3), glutathione peroxidases (GPX), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), and thioredoxin (TRX) family (TRX-1 and TRX-2) (Itoh et al. 1997; Cho et al. 2006; Riedl et al. 2009), as well as some non-enzyme substances, such as glutathione (GSH) and oxidized glutathione (GSSG). Moreover, it is reported that cells typically accomplish OS through the induction of detoxification enzymes by transcription factor Nrf2 (Wilhelmi et al. 2013). During OS, Nrf2 is released from its cytosolic repressor Keap1. The phosphorylated Nrf2 translocates into the nucleus where it binds to antioxidant response elements (AREs) residing within the promoter regions of many antioxidant and phase II genes (Lee et al. 2004). Among various phase II detoxification enzymes, HO-1 could be highly induced by a variety of agents causing OS (Choi et al. 1996). Hence, HO-1 can be used as an indicator for oxidative injury (Yachie et al. 1999).

Probiotics, defined as live microorganisms in adequate amounts conferring a health benefit on host (Araya 2002), have been reported to have antioxidant potential both in vitro and in vivo (Mishra et al. 2015). Probiotic *Bacillus*, such as *Bacillus subtilis*, may grow in the gut and consume oxygen to maintain an anaerobic environment for the prevention or treatment of gastrointestinal disorders (Hu et al. 2014). *Bacillus* has been focused on due to its higher resistance to harsh environments and capacity for long-term storage at ambient temperature (Hong et al. 2005). Recent researches have shown that probiotic *Bacillus* could enhance the antioxidant activity of RAW 264.7 cells (Li et al. 2013) and broilers (Rajput et al. 2013); however, few studies have elaborated its mechanisms of action of antioxidation. In this study, we employed seven strains of *Bacillus*, including *Bacillus subtillis*, *Bacillus natto*, *Bacillus lichenifoemis*, *Bacillus polymyxa*, *Bacillus coagulans*, and *Bacillus amyloliquefaciens*, to treat IPEC-1 alone or before H_2O_2 administration to investigate their antioxidant capacities, and we could further study its underlying antioxidant mechanisms.

Materials and methods

Bacteria strains and cell culture

B. subtillis SC02 was obtained from Huayu Agricultural Technology Company, China. *B. amyloliquefaciens* SC06, deposited in China Center For Type Culture Collection (CCTCC No. M 2012280), was isolated and preserved in our lab. *B. natto* SC07 was a gift from NABIO, Japan. *B. lichenifoemis* SC08 was isolated from a commercial product *Bacillus licheniformis* Capsule, China. *B. polymyxa* SC10 was isolated from the biopesticide Kangdeleide, China. *B. coagulans* SC12 was obtained from China General Microbiological Culture Collection Center, Beijing, China (CGMCC NO1.3220). *B. amyloliquefaciens* SC33 was a gift from Professer Shouwen Chen, Huazhong Agricultural University, China. All bacteria were cultured in Luria–Bertani broth at 37 °C for 12 h, and cells were collected after centrifugation at 5000 rpm for 10 min at 4 °C. Thereafter, these cells were washed twice with sterile phosphate-buffered saline (PBS, pH 7.2). Finally, their purity and identification were constantly checked by spreading plate method (Nikoskelainen et al. 2003).

IPEC-1 cell culture

IPEC-1 was obtained from the Cell Bank of the Chinese Academy of Sciences (CBCAS), Shanghai, China, and cultured in DMEM-F12 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Paisley, Scotland), 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in the incubator with 90% humidity and 5% CO_2, refed every 2 days, and passaged every 4 days.

Establishment of OS model

Cell viability was determined by methyl thiazolyl tetrazolium (MTT) assay. IPEC-1 (1×10^4 cells/well) were seeded into a 96-well plate (Corning, USA) overnight before treatments. The cells were then treated with H_2O_2 at different final concentrations (0, 100, 200, 300, 400, 500, 600, 700 μmol/L) for various time periods (1, 3, 6, 12 h), followed by culturing with 5 mg/mL of MTT working solution for 4 h at 37 °C. After incubated with 100 μL of DMSO for 10 min, the cells were measured using SpectraMax M5 (MD, USA) at the absorbance of 490 nm. Based on MTT assay, the median lethal dose (LD_{50}) of H_2O_2 was calculated by probability unit. Then, combined the MTT data with LD_{50}, the optimal concentration and incubation time of H_2O_2 were chosen to establish the OS model.

Co-culture of IPEC-1 with *Bacillus*

Cell suspension at a concentration of 5×10^5 cells/mL was seeded into 12-well culture plates (1 mL per well) (Corning, USA) and grown in a CO_2 incubator for 24 h. Thereafter, cells were washed three times with PBS. IPEC-1 in *Bacillus* treatment groups was co-cultured with 1×10^8 colony-forming unit (cfu) *Bacillus* in 1 mL complete DMEM-F12 medium without antibiotic for 3 h, and 1 mL of culture medium without antibiotics was used as control group. The concentration and treatment time of *Bacillus* used in our study (1×10^8 cfu/mL) were selected by co-culturing 1×10^6 cfu and 1×10^8 cfu SC06 with IPEC-1 for different time periods (3, 6, 9 h) (data not shown).

Cell treatments

To determine the antioxidant effects of *Bacillus*, IPEC-1 cells were divided into four groups: control, *Bacillus* treatment, H_2O_2 treatment, and *Bacillus* pre-protection + H_2O_2 treatment groups. Briefly, IPEC-1 cells in control group were cultured in complete DMEM-F12 medium (without antibiotic) for 3 h. And then, cells were washed with PBS and cultured in complete culture medium for another 12 h. For *Bacillus* group, all seven strains of *Bacillus* were co-cultured with IPEC-1 in culture medium (without antibiotic) for 3 h at first, and then, PBS was used to wash away probiotics. And complete medium was added to culture cells for another 12 h. For H_2O_2 group, IPEC-1 cells were cultured in medium (without antibiotic) for 3 h. Subsequently, complete medium containing H_2O_2 (optimal H_2O_2 concentration determined by MTT and LD_{50}) was added to treat cells for a certain time (determined by MTT and LD_{50}) to induce OS. In *Bacillus* pre-protection + H_2O_2 group, after IPEC-1 co-cultured with *Bacillus* for 3 h, PBS was used to wash away probiotics; then, cells were incubated with H_2O_2 under the same conditions as the H_2O_2 treatment group.

Biochemical assays for the antioxidant capacity of IPEC-1 cells

The GSSG, GSH levels, and CAT, SOD, GPX, and NOX activities in cell lysates were measured to determine the antioxidant capacity of IPEC-1. All assays were carried out following the manufacturer's instructions of commercial kits (Nanjing Jiancheng Bioengineering Institute, China).

Detection of ROS generation

ROS concentration in IPEC-1 was assayed using Reactive Oxygen Species Assay Kit (Beyotime Biotechnology, China) including dichlorfluorescein (DCFH)-DA (10 mol/L), which is sensitive to H_2O_2 and its derivatives, such as ·OH. Signals were monitored by SpectraMax M5 (MD, USA).

Detection of $\Delta\psi m$

JC-1 probe was employed to measure mitochondrial depolarization in IPEC-1 with Mitochondrial Membrane Potential Assay Kit (Beyotime Biotechnology, China). According to instruction of manufacturer, IPEC-1 cells were stained with JC-1 for 15 min at 37 °C. The monomeric form of JC-1 in the cytosol after mitochondrial membrane depolarization and the potential-dependent aggregation of JC-1 in the mitochondria were detected by SpectraMax M5 (MD, USA), respectively.

Detection of IPEC-1 apoptosis and necrosis

Cell apoptosis and necrosis were determined using Apoptosis and Necrosis Assay Kit (Beyotime Biotechnology, China), respectively. According to the manufacturer's instructions, IPEC-1 cells were stained with Hoechst 33342 (10 ng/mL) and propidium iodide (PI, 10 ng/mL) for 20 min at 4 °C in the dark, respectively. The condensed or fragmented nuclei of apoptotic cells were observed with a Leica fluorescence microscope (Keyence, Osaka, Japan).

RNA extraction and quantitative real-time PCR

Extraction of total RNA and its reverse transcription was performed according to our previous reports (Mao et al. 2015). PCR primer sequences for the pig genes were designed and selected by primer 5.0 and oligo 7.0 software as presented in Table S1 (online resource). *GAPDH* as housekeeping gene was used to normalize target gene transcript levels. Real-time PCR was performed using Premix Ex TaqTM with SYBR Green (TaKaRa, Dalian, China) and ABI Stepone Real-Time PCR System 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The thermocycle protocol includes 30-s at 95 °C followed by 40 cycles of 5-s denaturation at 95 °C, 34-s annealing/extension at 60 °C, and then a final melting curve analysis to monitor purity of the PCR product. The $2^{-\Delta\Delta Cq}$ method was used to estimate messenger RNA (mRNA) abundance. Relative gene expression levels were normalized by eukaryotic reference gene *GAPDH*.

Western blotting analysis

Total cell lysates were prepared as previously described (Majumdar et al. 1993). Nuclear and cytosolic extracts were fractionated using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology) according to the manufacturer's instructions. Equal amounts of proteins from each sample were subjected to SDS-PAGE, and then, proteins on the gel were transferred to nitrocellulose membranes. Membranes were blocked by no protein blocking solution (Sangon Biotech) and then incubated with the primary antibodies (anti-β-actin, anti-Nrf2, anti-phospho-Nrf2, anti-

Keap1, and anti-p47^{phox} antibodies) overnight at 4 °C. After washing with TBST, membranes were incubated with secondary antibody adjusted with HRP (Biotime Biotechnology, China). The blots were then developed with an ECL detection system according to the manufacturer's instructions.

Statistical analysis

LD_{50} of H_2O_2 to IPEC-1 was calculated using probit method in SPSS 16.0 for Windows. Results were expressed as mean ± standard deviation (SD). Data analysis was performed using one-way analysis of variance (ANOVA) procedure of SPSS by the Tukey test. Differences were considered statistically significant at $p < 0.01$ or 0.05.

Results

Effects of different concentrations of H_2O_2 on cell viability of IPEC-1

We first examined the cell viability of IPEC-1 using MTT method after treated with H_2O_2. H_2O_2 at different final concentrations (0, 100, 200, 300, 400, 500, 600, 700 μmol/L) was used to treat IPEC-1 cells for 1, 3, 6, and 12 h. Using the probit method, LD_{50} for H_2O_2 to IPEC-1 at 12 h was calculated to be 323.59 μmol/L. Moreover, Fig. 1 also shows that with the increase of H_2O_2 concentration and induction time, cell viability witnessed a decline and the treatment of IPEC-1 with 300 μmol/L H_2O_2 for 12 h reduced cell viability to 54.04 ± 1.34%. Thus, 300 μmol/L H_2O_2 was used to treat IPEC-1 for 12 h in order to induce the OS for the follow-up experiments.

Selection of an effective indicator for OS of IPEC-1 induced by H_2O_2

In order to choose an effective OS indicator, 300 μmol/L H_2O_2 was used to treat IPEC-1 cells for various time periods

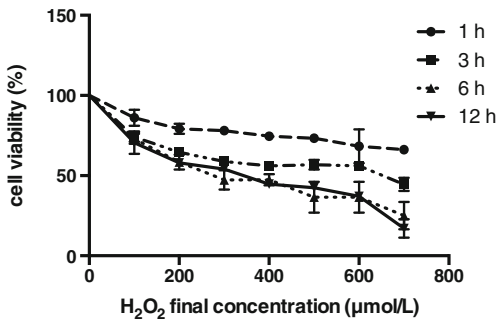

Fig. 1 Cell viability with H_2O_2 treatment. IPEC-1 cells were treated with different concentrations of H_2O_2 (0, 100, 200, 300, 400, 500, 600, 700 μmol/L) for 1, 3, 6, and 12 h. Cell viability was determined using MTT assay. Results are given as mean ± SD

(0, 1.5, 3, 6, 9, 12, and 18 h), and the mRNA expressions of various antioxidant and apoptosis genes were measured (Table S2, online resource). Results demonstrated that compared to other tested genes, *HO-1* was extremely sensitive to H_2O_2 stimulation. After 12-h 300 μmol/L H_2O_2 incubation, *HO-1* mRNA relative expression enhanced to 13.96 ± 1.31 (Table S2, online resource). Moreover, through the correlation analysis, we found that there was a good positive correlation under OS between *HO-1* expression and the H_2O_2 treatment time (R^2 = 0.9628) (Table S2, online resource). In previous study, it was confirmed that the induction of *HO-1* could represent an adaptive response to oxidative injury with increasing cell resistance (Martin et al. 2004). Hence, in the study, we chose *HO-1* as the primary indicator for OS in IPEC-1, and other antioxidant and apoptosis genes were also considered as secondary indicators.

Effects of *Bacillus* strains on OS of IPEC-1

In the study, we investigated the effects of *Bacillus* candidates on OS of IPEC-1 depending on the expressions of apoptosis and antioxidant genes, especially *HO-1*. As shown in Fig. 2, compared with the result in H_2O_2 group, SC06 pre-protection markedly decreased *HO-1* transcription by 41%, indicating the most favorable effect among seven strains. Additionally, in terms of other genes encoding antioxidant enzymes, such as *SOD-1*, *GPX-2*, *CAT*, *GST*, and *TRX-1*, SC02 significantly decreased *SOD-1* expression while increased *TRX* and *GST* expression compared to control group (Fig. 2a). SC06 significantly increased *CAT* and *GST* expressions (Fig. 2b). Besides, SC07 markedly decreased *CAT*, *SOD-1*, *GPX-2*, and *GST* transcript levels, while *TRX-1* was induced (Fig. 2c). On the contrary, SC08 enhanced the transcription of *CAT* and *SOD-1* (Fig. 2d). Although SC10 also elevated *SOD-1* level, it could decrease *GST* gene expression as well (Fig. 2e). However, SC12 did not show significant effects on tested antioxidant genes (Fig. 2f), and SC33 regulated only *GST* mRNA expression (Fig. 2g). Despite of this, compared with H_2O_2 groups, *Bacillus* pre-protection also significantly altered antioxidant gene expression profiles. SC02 pre-treatment significantly increased the expressions of *CAT* and *TRX-1* and decreased the expressions of *HO-1* and *GPX-2* (Fig. 2a). SC06 pre-protection not only downregulated *SOD-1*, *GPX-2*, and *TRX-1* expressions but also upregulated *GST* transcript level (Fig. 2b). However, SC07 pre-treatment could markedly downregulate the transcript levels of *HO-1*, *GPX-2*, and *TRX-1* (Fig. 2c). Meanwhile, SC08 pre-protection decreased *GPX-2* and *TRX-1* transcriptions (Fig. 2d). Expressions of *SOD-1*, *HO-1* as well as *GPX-2* were downregulated by SC10 pre-protection (Fig. 2e). Moreover, *GPX-2* expressions were significantly decreased with SC12 and SC33 pre-treatment, but *CAT* transcriptions were upregulated by SC33 pre-treatment (Fig. 2f, g).

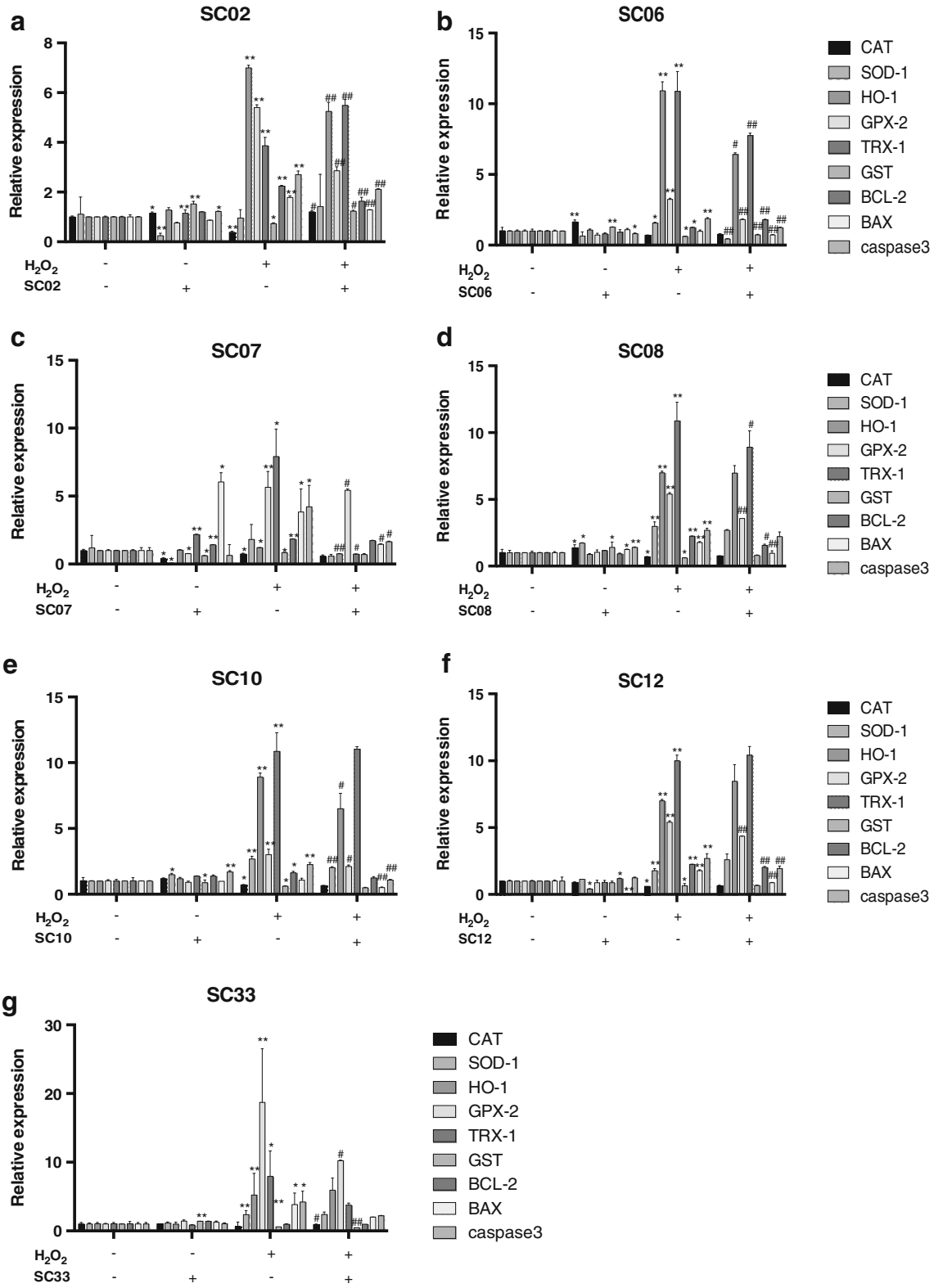

Fig. 2 Effects of seven *Bacillus* strains on the expressions of genes related to antioxidant capacity and apoptosis in IPEC-1. Cells were co-cultured with *Bacillus* for 3 h; thereafter, 300 μmol/L H_2O_2 was added for 12 h. Gene expressions of *CAT*, *SOD-1*, *HO-1*, *GPX-2*, *TRX-1*, *GST*, *BCL-2*, *BAX*, and *caspase 3* were detected by real-time PCR. Results are given as mean ± SD. Differences between groups were determined by one-way ANOVA followed by Tukey test ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to the control group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as compared to the H_2O_2-treated group

As for the apoptosis, SC02, SC07, SC08, and SC10 could significantly upregulate pro-apoptotic gene expressions individually (Fig. 2a–e). However, SC12 downregulated *BAX* transcription and upregulated *BCL-2* expression, while SC33 has not shown effect on apoptosis-related genes (Fig. 2f). Notably, SC06 significantly decreased *caspase 3* expression, and the pre-protection with SC06 remarkably upregulated anti-apoptotic gene expression and downregulated pro-apoptotic gene expressions (Fig. 2b). Altogether, SC06 could efficiently protect IPEC-1 from OS and apoptosis. Thus, SC06 was chosen for further study to investigate the mechanisms of action of antioxidation.

Effects of SC06 on antioxidant parameters in cell lysates of IPEC-1

In cell lysates, we observed that H_2O_2 significantly decreased GSH/GSSG ratio but increased CAT and SOD activities. However, SC06 alone significantly elevated both GSH/GSSG ratio and CAT activity compared with non-treated group. Moreover, SC06 pre-protection could decrease CAT, SOD, and GPX activities compared with H_2O_2 group (Table 1).

Effects of SC06 on Nrf2/Keap1 signaling pathway of IPEC-1

As shown in Fig. 2b, *CAT* and *GST* expressions were increased in SC06-treated IPEC-1. mRNA expressions of *SOD-1*, *HO-1*, *GPX-2*, *GST*, and *TRX* were decreased in SC06 pre-protected cells. SOD, HO-1, GPX, GST, and TRX are well-known Nrf2 target genes (Itoh et al. 1997; Cho et al. 2006; Riedl et al. 2009). These findings implicated that SC06 could play a role in Nrf2/Keap1 signaling pathway. Since Nrf2 has a notably short half-life of approximately 20 min but possibly as long as 3 h (Sekhar et al. 2002; Kwak et al. 2003), thus, the IPEC-1 cells were sampled to detect Nrf2/Keap1 signaling transduction after co-culturing with 300 μmol/L H_2O_2 for different time periods (0, 30, 60, and 120 min). Compared with the control group, 300 μmol/L H_2O_2 incubation for 30, 60, and 120 min induced higher Nrf2 phosphorylation (Fig. 3b–f). Moreover, co-culture with SC06 for 3 h also increased Nrf2 expression and phosphorylation in IPEC-1 (Fig. 3a–f). On the contrary, SC06 pre-protection significantly decreased Nrf2 phosphorylation with the stimulation of H_2O_2 for 30 and 60 min (Fig. 3b–d, f). At 120 min, there was no significantly different phosphorylation level of Nrf2 between H_2O_2 and SC06 pre-protection + H_2O_2 group (Fig. 3e, f). Keap1 is the inhibitor of Nrf2; here, we did not observe significant expression change of Keap1 among different groups at different time points (Fig. 3).

Effects of SC06 on ROS production of IPEC-1

H_2O_2 significantly increased ROS concentrations in IPEC-1, while SC06 pre-protection dramatically decreased ROS levels. However, there was no significant difference in ROS levels between control group and SC06 group (Fig. 4a). $p47^{phox}$ is one of the active subunits of NOX, which plays an important role in ROS production. As shown in Fig. 4b, there was no significant change in $p47^{phox}$ expression after incubation with H_2O_2 for 12 h. In contrast, SC06 alone or pre-protection significantly decreased $p47^{phox}$ expression. Additionally, NOX activity was also significantly downregulated by SC06 pre-protection (Fig. 4c).

Effects of SC06 on Δψm of IPEC-1

Unstable Δψm can occur via mechanisms involving ROS-induced ROS release (Zorov et al. 2000). To further investigate ROS production, Δψm was determined. As shown in Fig. 5, after H_2O_2 administration, the ratio of J-aggregates and J-monomer in IPEC-1 experienced a slight down trend. Whereas, with SC06 pre-protection, the ratio of J-aggregates to J-monomer was increased, indicating the improvement of Δψm.

Effects of SC06 on IPEC-1 apoptosis and necrosis

Consistent with downregulated apoptosis-related genes observed in SC06 pre-protection group, we found that H_2O_2 incubation significantly increased IPEC-1 apoptosis (PI) and necrosis (Hoechst 33342), while with SC06 pre-protection, both the apoptosis and necrosis were ameliorated (Fig. 6).

Table 1 Effects of SC06 on antioxidant parameters in cell lysate

Parameters	Control	SC06	H2O2	SC06 + H2O2
GSH/GSSG	2.58 ± 0.03	3.86 ± 0.11**	1.67 ± 0.39*	2.15 ± 0.05
CAT (U/mgprot)	3.81 ± 0.06	5.82 ± 0.51*	6.68 ± 0.32**	2.86 ± 0.85##
SOD (U/mgprot)	22.07 ± 0.49	22.33 ± 2.33	29.78 ± 2.88*	15.32 ± 0.41#
GPX (U/mgprot)	185.54 ± 56.27	226.59 ± 33.46	684.96 ± 17.32**	164.72 ± 30.27##

IPEC-1 were co-cultured with *Bacillus* (strain SC06) for 3 h; thereafter, 300 μmol/L H_2O_2 was added for 12 h. Results are given as mean ± SD. Differences between groups were determined by one-way ANOVA followed by Tukey test ($n = 4$)

*$p < 0.05$ and **$p < 0.01$ as compared to the control group, #$p < 0.05$ and ##$p < 0.01$ as compared to the H_2O_2-treated group

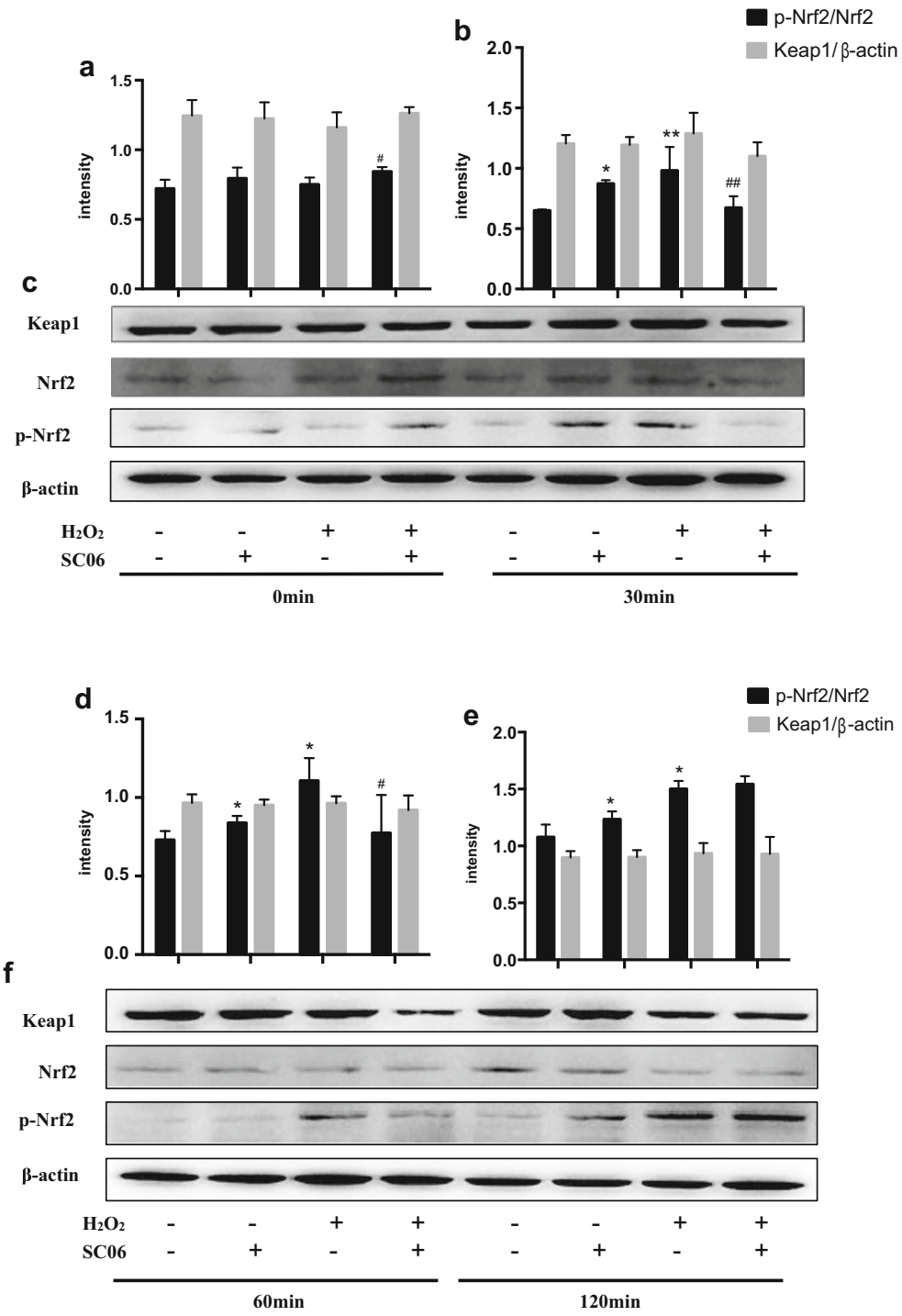

Fig. 3 Effects of SC06 on Nrf2/Keap1 signaling pathway in IPEC-1. Cells were co-cultured with SC06 for 3 h; thereafter, 300 μmol/L H_2O_2 was added for 0 min (**a**), 30 min (**b**), 60 min (**d**), and 120 min (**e**), respectively. Phosphorylated and total protein levels of Nrf2, Keap1, and β-actin in IPEC-1 cells were determined using Abs recognizing phospho-specific or total protein (**c**, **f**). Results are given as mean ± SD. Differences between groups were determined by one-way ANOVA followed by Tukey test ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to the control group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as compared to the H_2O_2-treated group

Discussion

Previous studies have proved that OS was involved in epithelial barrier injury (Sun et al. 2002). Hydroperoxides have been shown to elicit apoptosis and mucosal cytostasis in association with GSH/GSSG disruption in CaCo-2 cells and rat intestine (Gotoh et al. 2002; Tsunada et al. 2003a,b). Hence, the redox homeostasis in intestinal epithelium is critical to keep animal health. Accumulating evidence demonstrated that probiotics exerted various beneficial effects, one of them being the antioxidant ability. Although reports have shown the antioxidant capacity of some probiotic strains (Tovar-Ramírez et al. 2010; Amaretti et al. 2013), there was little evidence on oxidation-resistant effects and its MOA of *Bacillus*. Thus, in this study, we aimed to study the MOA of antioxidation using a strain of *Bacillus* with antioxidant capacity.

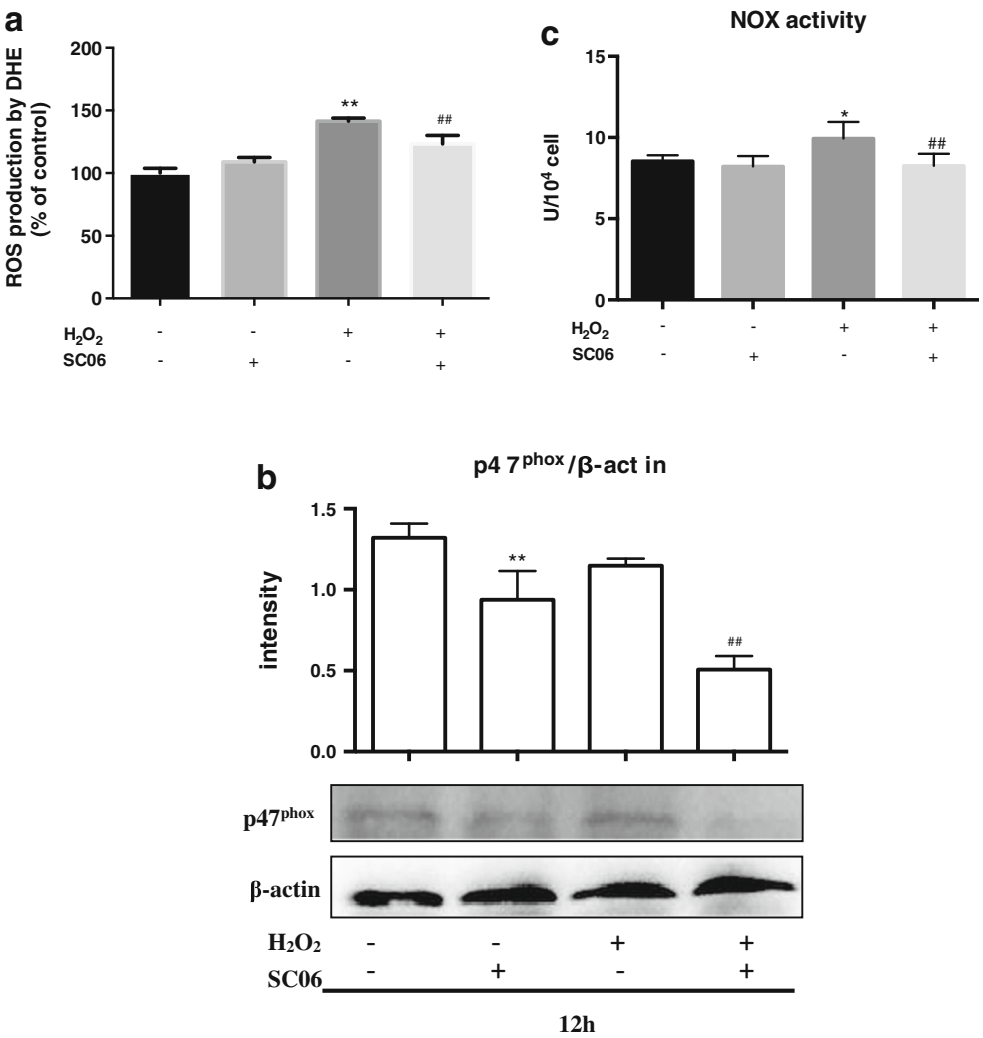

Fig. 4 Effects of SC06 on ROS production in IPEC-1. Cells were co-cultured with *Bacillus* (strain SC06) for 3 h; thereafter, 300 μmol/L H_2O_2 was added for 12 h. **a** ROS levels in IPEC-1 measured by DCFH. **b** NADPH oxidase activity. **c** Western blot results for $p47^{phox}$ expression. Results are given as mean ± SD. Differences between groups were determined by one-way ANOVA followed by Tukey test ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to the control group, $^{\#\#}p < 0.01$ as compared to the H_2O_2-treated group

H_2O_2, a strong oxidant capable of oxidizing a variety of moieties, was used to establish OS model in this study as it is yet not highly destructive (Ji 2007). The results from MTT assay and the LD_{50} suggested that H_2O_2 (300 μmol/L for 12 h) was sufficient to induce OS in IPEC-1 (Fig. 1). It is well known that HO-1 isozyme is a phase II enzyme that is transcriptionally regulated by a large variety of stimuli (Bauer and Bauer 2002; Ryter and Choi 2002; Scapagnini et al. 2002; Salinas et al. 2003). In this study, according to phase II gene expression level and its correlation with treatment time, we also found that *HO-1* was extremely sensitive to H_2O_2 induction and there was a positive correlation between *HO-1* transcript level and incubation time (Table S2, online resource), implying that *HO-1* could be a sensitive indicator for OS. As for the screening of *Bacillus* with potent antioxidant ability, we found that several *Bacillus* candidates by pre-treatment downregulated *HO-1* transcription markedly compared to that in H_2O_2 group, and SC06 had the most favorable effect (Fig. 2b), implying that SC06 may possess strong antioxidant ability. Usually, in malondialdehyde, protein carbonyl levels, and 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reduction activity potential (FRAP) methods were used to measure the OS (Sekher Pannala et al. 2001; Pirinccioglu et al. 2010; Clarke

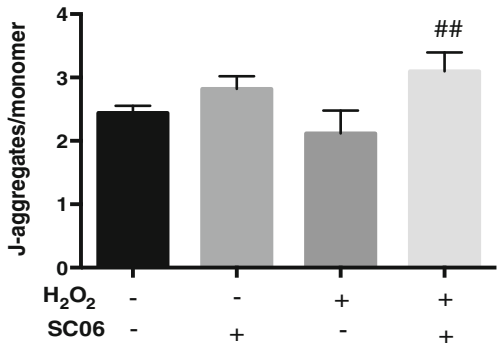

Fig. 5 Effects of SC06 on Δψm in IPEC-1. Cells were co-cultured with *Bacillus* (strain SC06) for 3 h; thereafter, 300 μmol/L H_2O_2 was added for 12 h. Results are given as mean ± SD. Differences between groups were determined by one-way ANOVA followed by Tukey test ($n = 3$). $^{\#\#}p < 0.01$ as compared to the H_2O_2-treated group

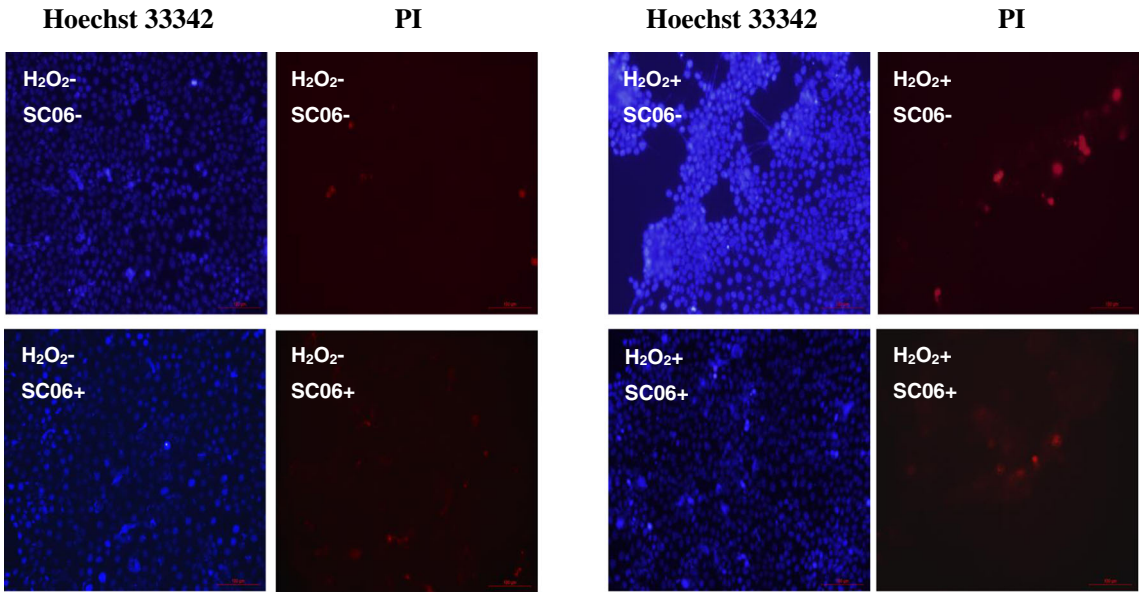

Fig. 6 Effects of SC06 on apoptosis and necrosis in IPEC-1. Cells were co-cultured with *Bacillus* (strain SC06) for 3 h; thereafter, 300 μmol/L H_2O_2 was added for 12 h. Then, IPEC-1 cells were stained with Hoechst 33342 and PI. Cell images were obtained under a fluorescence microscope (×100)

et al. 2013; Lei et al. 2015), whereas these methods have some disadvantages, for example, DPPH could be affected by light, oxygen, and pH (Ozcelik et al. 2003), and FRAP also has some drawbacks such as interference, reaction kinetics, and quantitation methods (Ou et al. 2002). Therefore, detecting the *HO-1* expression could be an easy method in evaluating OS. Thereafter, we also determined the expression levels of other antioxidant and apoptosis-related genes. The results demonstrated that SC06 alone could increase *CAT* and *GST* expressions to elevate antioxidant status, while SC06 pre-protection significantly downregulated *SOD-1*, *GPX-2*, and *TRX-1* but upregulated *GST* transcript level. Furthermore, no matter H_2O_2 pre-induction or not, SC06 addition was efficient in reducing pro-apoptotic genes of IPEC-1 cells. All together, *B. amyloliquefaciens* SC06 was able to modulate IPEC-1 antioxidant status dramatically, and to our knowledge, this study firstly proved that *HO-1* was an effective indicator for OS induced by H_2O_2 in IPEC-1 cells and may be used as a sensitive target to screen antioxidants.

To further investigate the antioxidant mechanisms of SC06, we measured GSH/GSSG ratio and antioxidase activities in IPEC-1 lysates. Compared with control, after co-culture with SC06, there was a significantly higher GSH/GSSG value and SOD activity, while SC06 pre-protection significantly decreased CAT, SOD, and GPX activities compared to that under OS (Table 1). *HO-1*, *CAT*, *SOD-1*, *GPX-2*, *GST*, and *TRX-1* are phase II genes, and it is known that phase II detoxification enzymes as well as glutathione synthesis can be regulated via Nrf2/Keap1 signaling pathway (Maher and Yamamoto 2010). So, the altered phase II gene expressions, glutathione system, and antioxidase activities implicated the effects of SC06 on Nrf2/Keap1 signaling pathway. And we found that SC06 alone was able to phosphorylate Nrf2. Furthermore, H_2O_2 also increased the phosphorylation of Nrf2 in IPEC-1 (Fig. 3) because when cells were exposed to oxidants and electrophiles, Nrf2 could be activated (Ma et al. 2013) to protect cells against OS. Interestingly, with SC06 pre-protection, H_2O_2 would not activate Nrf2 anymore but downregulated Nrf2 phosphorylation at 30 and 60 min (Fig. 3), which was in line with the downregulated phase II gene expressions and antioxidase activities in SC06 pre-protection + H_2O_2 treatment. However, several reports have revealed that under OS, probiotic administration could induce Nrf2 and its targeted antioxidase (Endo et al. 2013; Gao et al. 2013) rather than suppressing them. In the study, after 3-h pre-protection with SC06, the Nrf2/Keap1 signaling pathway had been activated already, leading to the increased phase II gene expressions and antioxidase activities. In 2005, Kobayashi and Yamamoto reported that the activation of the defense system by phase 2 induction renders cells more resistant to the potential challenges of a subsequent, even greater stress. So here, the enhanced antioxidant status of IPEC-1 caused by SC06 could make IPEC-1 more resistant to the potential challenge of H_2O_2, and it is not necessary for IPEC-1 cells to continue upregulating Nrf2 phosphorylation and increasing antioxidase activities after the subsequent H_2O_2 treatment.

Besides the antioxidant signaling, we also determined the ROS production in IPEC-1. ROS are by-products of aerobic metabolism and are often associated with the principle of OS (Schieber et al. 2014). NOX are proteins transferring electrons across biological membranes to generate superoxide and other

downstream ROS (Bedard and Krause 2007). Among all NOX subunits, $p47^{phox}$ is considered to be responsible for transporting the cytosolic complex to the membrane during oxidase activation (Clark et al. 1990; Quinn et al. 1993; El-Benna et al. 1994) and is considered as the organizer of the NOX active complex (El-Benna et al. 2008). Our results implied that the increased ROS concentration and NOX activity induced by H_2O_2 were effectively reduced by SC06 pre-protection (Fig. 4a, c). Moreover, the results from western blotting also suggested that SC06 alone could downregulate the protein expression of NOX active subunit $p47^{phox}$ compared with control group. Similarly, SC06 pre-protection also significantly decreased $p47^{phox}$ level compared with the H_2O_2 group (Fig. 4b). In summary, SC06 could ameliorate OS and enhance cell antioxidant status in IPEC-1 through activating Nrf2/Keap1 signaling transduction and inhibiting ROS generation.

From a quantitative viewpoint, mitochondria are thought to be the largest contributors to intracellular oxidant production in most cell types (Holmstrom et al. 2014). Although it is widely accepted that mitochondria produce more ROS at high membrane potential, in certain pathological conditions, there were opposite correlations between $\Delta\psi m$ and ROS production (Suski et al. 2012). In the case of mitochondrial disorders associated with the dysfunctions of the respiratory chain components, lower $\Delta\psi m$ is observed with an increase in ROS production (Lebiedzinska et al. 2010). In the present study, H_2O_2 slightly decreased $\Delta\psi m$, while SC06 pre-protection could enhance the $\Delta\psi m$ (Fig. 5) and decrease ROS generation simultaneously. Besides, mitochondrial dysfunction has been shown to participate in the induction of apoptosis (Ly et al. 2003), and the loss of $\Delta\psi m$ may be an early event in the apoptotic process. In this study, as the IPEC-1 $\Delta\psi m$ slightly decreased in H_2O_2 group (Fig. 5), apoptosis and necrosis were also induced (Figs. 2b and 6); however, SC06 pre-protection lowered IPEC-1 apoptosis and necrosis markedly (Figs. 2b and 6).

In conclusion, our study proves that *HO-1* can be an ideal indicator for OS model induced by H_2O_2 and confirms that SC06 has the potent antioxidant capacity. Pre-protection with SC06 can effectively regulate Nrf2/Keap1 signaling pathway and decrease NOX expression as well as activity. As a result, SC06 improves IPEC-1 antioxidant status and reduces apoptosis as well as necrosis. Our results suggested that *B. amyloliquefaciens* SC06 could serve as a potential prophylactic nutrient additive to protect porcine intestinal cells from OS. The findings may also provide important implications for improving human gut health.

Acknowledgements This study is supported by the National High-Tech R&D Program (863) of China (No. 2013AA102803D), the National Natural Science Foundation of China (No. 31472128), and the Major Science and Technology Project of Zhejiang Province (No. 2006C12086), China.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The research performed did not involve human participants and/or animals.

References

Amaretti A, di Nunzio M, Pompei A, Raimondi S, Rossi M, Bordoni A (2013) Antioxidant properties of potentially probiotic bacteria: in vitro and in vivo activities. Appl Microbiol Biotechnol 97: 809–817

Araya M (2002) Guidelines for the evaluation of probiotics in food. Report of a Joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. London Ontario, Canada

Bauer M, Bauer I (2002) Heme oxygenase-1: redox regulation and role in the hepatic response to oxidative stress. Antioxid Redox Signal 4:749–758

Bedard K, Krause KH (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87:245–313

Cho HY, Reddy SP, Kleeberger SR (2006) Nrf2 defends the lung from oxidative stress. Antioxid Redox Signal 8:76–87

Choi AM, Alam J (1996) Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Respir Cell Mol Biol 15:9–19

Circu ML, Aw TY (2012) Intestinal redox biology and oxidative stress. Semin Cell Dev Biol 23:729–737

Clark RA, Volpp BD, Leidal KG, Nauseef WM (1990) Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. J Clin Invest 85:714–721

Clarke G, Ting KN, Wiart C, Fry J (2013) High correlation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric reducing activity potential and total phenolics content indicates redundancy in use of all three assays to screen for antioxidant activity of extracts of plants from the malaysian rainforest. Antioxidants 2:1–10

El-Benna J, Ruedi JM, Babior BM (1994) Cytosolic guanine nucleotide-binding protein Rac 2 operates in vivo as a component of the neutrophil respiratory burst oxidase. Transfer of Rac 2 and the cytosolic oxidase components p47 (phox) and p67 (phox) to the submembranous actin cytoskeleton during oxidase activation. J Biol Chem 269:6729–6734

El-Benna J, Dang PM, Gougerot-Pocidalo MA (2008) Priming of the neutrophil NADPH oxidase activation: role of p47phox phosphorylation and NOX2 mobilization to the plasma membrane. Semin Immunopathol 30:279–289

Endo H, Niioka M, Kobayashi N, Tanaka M, Watanabe T (2013) Butyrate-producing probiotics reduce nonalcoholic fatty liver disease progression in rats: new insight into the probiotics for the gut-liver axis. PLoS One 8:e63388

Gao D, Gao Z, Zhu G (2013) Antioxidant effects of *Lactobacillus plantarum* via activation of transcription factor Nrf2. Food Funct 4:982–989

Gotoh Y, Noda T, Iwakiri R, Fujimoto K, Rhoads CA, Aw TY (2002) Lipid peroxide-induced redox imbalance differentially mediates CaCo-2 cell proliferation and growth arrest. Cell Prolif 35:221–235

Holmstrom KM, Finkel T (2014) Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat Rev Mol Cell Biol 15:411–421

Hong HA, Duc LH, Cutting SM (2005) The use of bacterial spore formers as probiotics. FEMS Microbiol Rev 29:813–835

Hu Y, Dun Y, Li S, Zhao S, Peng N, Liang Y (2014) Effects of *Bacillus subtilis* KN-42 on growth performance, diar rhea and faecal bacterial flora of weaned piglets. Asian Australas J Anim Sci 27:1131–1114

Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yama moto M, Nabeshima Y (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 236:313–322

Ji LL (2007) Antioxidant signaling in skeletal muscle: a brief review. Exp Gerontol 42:582–593

Kobayashi M, Yamamoto M (2005) Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. Antioxid Redox Signal 7:385–394

Kwak MK, Wakabayashi N, Itoh K, Motohashi H, Yamamoto M, Kensler TW (2003) Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway. Identification of novel gene clusters for cell survival. J Biol Chem 278:8135–8145

Lambeth JD (2004) NOX enzymes and the biology of reactive oxygen. Nat Rev Immuno 4:181–189

Lebiedzinska M, Karkucinska-Wieckowska A, Giorgi C, Karczmarewicz E, Pronicka E, Pinton P, Duszyński J, Pronicki M, Wieckowski MR (2010) Oxidative stress-dependent p66Shc phosphorylation in skin fibroblasts of children with mitochondrial disorders. Biochim Biophys Acta 1797:952–960

Lee JM, Johnson JA (2004) An important role of Nrf2-ARE pathway in the cellular defense mechanism. J Biochem Mol Biol 37:139–143

Lei K, Li YL, Wang Y, Wen J, Wu HZ, Yu DY, Li WF (2015) Effect of dietary supplementation of *Bacillus subtilis* B10 on biochemical and molecular parameters in the serum and liver of high-fat diet-induced obese mice. J Zhejiang Univ Sci B 16:487–495

Li YL, Lei K, Xu X, Rajput IR, Yu DY, Li WF (2013) Protective effect of *Bacillus subtilis* B10 against hydrogen peroxide-induced oxidative stress in a murine macrophage cell line. Int J Agric Biol 15:927–932

Ly JD, Grubb DR, Lawen A (2003) The mitochondrial membrane potential ($\Delta\psi m$) in apoptosis: an update. Apoptosis 8:115–128

Ma Q (2013) Role of nrf2 in oxidative stress and toxicity. Annu Rev Pharmacol Toxicol 53:401–426

Maher J, Yamamoto M (2010) The rise of antioxidant signaling-the evolution and hormetic actions of Nrf2. Toxicol Appl Pharmacol 244:4–15

Majumdar S, Kane LH, Rossi MW, Volpp BD, Nauseef WM, Korchak HM (1993) Protein kinase C isotypes and signal-transduction in human neutrophils: selective substrate specificity of calcium-dependent beta-PKC and novel calcium-independent nPKC. Biochim Biophys Acta 1176:276–286

Mao Y, Wang B, Xu X, Du W, Li W, Wang Y (2015) Glycyrrhizic acid promotes M1 macrophage polarization in murine bone marrow-derived macrophages associated with the activation of JNK and NF-κB. Mediat Inflamm 2015

Martin D, Rojo AI, Salinas M, Diaz R, Gallardo G, Alam J, De Galarreta CM, Cuadrado A (2004) Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. J Biol Chem 279:8919–8929

Mishra V, Shah C, Mokashe N, Chavan R, Yadav H, Prajapati J (2015) Probiotics as potential antioxidants: a systematic review. J Agricultural Food Chem 63:3615–3626

Nikoskelainen S, Ouwehand AC, Bylund G, Salminen S, Lilius EM (2003) Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). Fish Shellfish Immunol 15:443–452

Ou B, Huang D, Hampsch-Woodill M, Flanagan JA, Deemer EK (2002) Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. J Agric Food Chem 50:3122–3128

Ozcelik B, Lee JH, Min DB (2003) Effects of light, oxygen, and pH on the absorbance of 2,2-diphenyl-1-picrylhydrazyl. J Food Sci 68: 487–490

Park JB (2003) Phagocytosis induces superoxide formation and apoptosis in macrophages. Exp Mol Med 35:325–335

Pirincciogl u AG, Gökalp D, Pirincciogl u M, Kizil G, Kizil M (2010) Malondialdehyde (MDA) and protein carbonyl (PCO) levels as biomarkers of oxidative stress in subjects with familial hypercholesterolemia. Clin Biochem 43:1220–1224

Quinn MT, Evans T, Loetterle LR, Jesaitis AJ, Bokoch GM (1993) Translocation of Rac correlates with NADPH oxidase activation. Evidence for equimolar translocation of oxidase components J Biol Chem 268:20983–20987

Rajput IR, Li LY, Xin X, Wu BB, Juan ZL, Cui ZW, Yu DY, Li WF (2013) Effect of *Saccharomyces boulardii* and *Bacillus subtilis* B10 on intestinal ultrastructure modulation and mucosal immunity development mechanism in broiler chickens. Poult Sci 92:956–965

Riedl MA, Saxon A, Diaz-Sanchez D (2009) Oral sulforaphane increases phase II antioxidant enzymes in the human upper airway. Clin Immunol 130:244–251

Ryter SW, Choi AMK (2002) Heme oxygenase-1: molecular mechanisms of gene expression in oxygen-related stress. Antioxid Redox Signal 4:625–632

Salinas M, Diaz R, Abraham NG, Ruiz de Galarreta CM, Cuadrado A (2003) Nerve growth factor protects against 6-hydroxydopamine-induced oxidative stress by increasing expression of heme oxygenase-1 in a phosphatidylinositol 3-kinase-dependent manner. J Biol Chem 278:13898–13904

Scapagnini G, Foresti R, Calabrese V, Giuffrida Stella AM, Green CJ, Motterlini R (2002) Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers. Mol Pharmacol 61:554–561

Schieber M, Chandel NS (2014) ROS function in redox signaling and oxidative stress. Curr Biol 24:R453–R462

Sekhar KR, Yan XX, Freeman ML (2002) Nrf2 degradation by the ubiquitin proteasome pathway is inhibited by KI AA0132, the human homolog to INrf2. Oncogene 21:6829–6834

Sekher Pannala A, Chan TS, O'Brien PJ, Rice-Evans CA (2001) Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. Biochem Biophys Res Commun 282:1161–1168

Sies H (1997) Oxidative stress: oxidants and antioxidants. Exp Physiol 82:291–295

Sinha K, Das J, Pal PB, Sil PC (2013) Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. Arch Toxicol 87:1157–1180

Sun Z, Olanders K, Lasson A, Dib M, Annborn M, Andersson K, Wang X, Andersson R (2002) Effective treatment of gut barrier dysfunction using an antioxidant, a PAF inhibitor, and monoclonal antibodies against the adhesion molecule PECAM-1. J Surg Res 105:220–233

Suski JM, Lebiedzinska M, Bonora M, Pinton P, Duszynski J, Wieckowski MR (2012) Relation between mitochondrial membrane potential and ROS formation. Methods Mol Biol 810:183–205

Tovar-Ramírez D, Mazurais D, Gatesoupe JF, Quazuguel P, Cahu CL, Zambonino-Infante JL (2010) Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (*Dicentrarchus labrax*) larvae. Aquaculture 300:142–147

Tsunada S, Iwakiri R, Fujimoto K, Aw TY (2003a) Chronic lipid hydroperoxide stress suppresses mucosal proliferation in rat intestine: potentiation of ornithine decarboxylase activity by epidermal growth factor. Dig Dis Sci 48:2333–2341

Tsunada S, Iwakiri R, Noda T, Fujimoto K, Fuseler J, Rhoads CA, Aw TY (2003b) Chronic exposure to subtoxic levels of peroxidized lipids

suppresses mucosal cell turnover in rat small intestine and reversal by glutathione. Dig Dis Sci 48:210–222

Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 160:1–40

Wilhelmi V, Fischer U, Weighardt H, Schulze-Osthoff K, Nickel C, Stahlmecke B, Kuhlbusch TA, Scherbart AM, Esser C, Schins RP, Albrecht C (2013) Zinc oxide nanoparticles induce necrosis and apoptosis in macrophages in a p47phox-and Nrf2-independent manner. PLoS One 8:e65704

Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S (1999) Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. J Clin Invest 103:129

Zhang JX, Guo LY, Feng L, Jiang WD, Kuang SY, Liu Y, Hu K, Jiang J, Li SH, Tang L, Zhou XQ (2013) Soybean β-conglycinin induces inflammation and oxidation and causes dysfunction of intestinal digestion and absorption in fish. PLoS One 8:e58115

Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ (2000) Reactive oxygen species (Ros-induced) Ros release a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. J Exp Med 192:1001–1014