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Glutathione biosynthesis is essential for antioxidant and anti-inflammatory effects of *Streptococcus thermophilus*

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ABSTRACT

Glutathione biosynthesis is catalysed by glutathione synthetase (GshF) in *Streptococcus thermophilus*. Here, the *gshF* mutant constructed from *S. thermophilus* ST2017 was sensitive to oxidative stress and decreased the viability of HT-29 cells under H_2O_2 exposure. Co-incubating the HT-29 cells with the *gshF* mutant attenuated the enhanced intracellular activities of the antioxidant enzymes of strain ST2017 under 2 mM H_2O_2 and decreased the transcription levels of Nrf2-related antioxidant enzyme genes. To investigate the anti-inflammatory effect, HT-29 cells were co-incubated with *S. thermophilus* strains, then challenged with *Salmonella braenderup* H9812. ELISA and RT-qPCR analysis showed that the *gshF* mutant could not effectively inhibit the pro-inflammatory cytokine production triggered by *Salmonella*. The results suggested that glutathione biosynthesis by GshF contributes to the antioxidant and anti-inflammatory effects of *S. thermophilus* ST2017 on its host cells.

1. Introduction

Streptococcus thermophilus is most frequently used in fermenting yoghurt and cheeses and is a “generally recognised as safe” (GRAS) species according to the American Food and Drug Administration. As the second most important species after *Lactococcus lactis* in the dairy industry, *S. thermophilus* possesses high milk fermentation capabilities and flavour producing characteristics (Hols et al., 2005; Nebesny & Żyżelewicz, 2006).

In addition to its use in dairy industry, several benefits of *S. thermophilus* have been studied (Naidu, Bidlack, & Clemens, 1999; Pagnini et al., 2010). *S. thermophilus* possesses a β-galactosidase, which catalyses lactose degradation, rendering health benefits in lactose digestion to lactose intolerant individuals, thus enabling better use of the nutrients in milk (Rabot, Rafter, Rijkers, Watzl, & Antoine, 2010; Savaiano, 2014). With long-term adaptation to the fermented dairy environment and transit in the human gastrointestinal tract (GIT), *S. thermophilus* possesses several biological functions necessary to survive through the GIT, including bile salt and acid tolerance and the ability to adhere to the host's intestinal tissues (Brigidi, Swennen, Vitali, Rossi, & Matteuzzi, 2003; Elli et al., 2006; Kebouchi et al., 2016; Mater et al., 2005). Although the antioxidant and anti-inflammatory activities of *S. thermophilus* have been debated (Amaretti et al., 2013; Junjua et al., 2016), their mechanisms of action have rarely been reported.

Glutathione (γ-glutamyl-cystemyl-glycine, GSH), composed of glutamate, cysteine and glycine, is an important non-protein thiol compound widely distributed in eukaryotes and Gram-negative organisms and sporadically distributed in Gram-positive bacteria (Pophaly, Singh, Pophaly, Kaushik, & Tomar, 2012). GSH is a key factor in keeping the intracellular redox homeostasis and protecting cells against oxidative damage (Li, Hugenholtz, Abee, & Molenaar, 2003). GSH is also vital for detoxifying xenobiotics, and regulating cell proliferation, apoptosis, immune function, and fibrogenesis (Wu, Fang, Yang, Lupton, & Turner, 2004). GSH biosynthesis involves two reactions that form peptide bonds. The first reaction between glutamate and cysteine is catalysed by γ-glutamylcysteine synthetase (GshA), and the second reaction between γ-Glu-Cys and glycine is catalysed by glutathione synthetase (GshB) (Copley & Dhillon, 2002). Alternately, several Gram-positive bacteria have evolved a bifunctional glutathione synthetase (GshF), which catalyses both reactions for glutathione biosynthesis in one step (Borgo, Carpen, Ferrario, Iametti, & Fortina, 2013; Li, Li, Yang, & Ye, 2011; Vergauwen, de Vos, & van Beeumen, 2006). We recently found that glutathione biosynthesis by GshF occurred extensively in *S. thermophilus* and was an important

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process that enabled this species to tolerate oxidative and acid stress (Wang, Lu, Lu, & Kong, 2015; Wang, Xu, Lu, Xin, & Kong, 2016). Although the effect of glutathione biosynthesis in *S. thermophilus* ST2017 defending host cells against oxidative stress has been well studied (Wang et al., 2017), the antioxidant effect of this strain, which depends on glutathione biosynthesis in intestinal epithelial cells, remains unclear. In this study, we created a glutathione synthetase (*gshF*) mutant and investigated the effect of glutathione biosynthesis in *S. thermophilus* ST2017 on antioxidant and anti-inflammatory effects in intestinal epithelial cells.

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. thermophilus ST2017, originally isolated from a traditional yoghurt in Inner Mongolia, China, and its *gshF* inactivated mutant were used in this study. *Lactobacillus crispatus* K313 was included as a positive control for the anti-inflammatory assays (Sun et al., 2012). *S. thermophilus* strains were propagated in M17 broth (Oxoid, Basingstoke, United Kingdom) supplemented with 1% (w/v) lactose (LM17 broth) at 42 °C without shaking. Erythromycin at 5 μg mL^{-1} was added to the LM17 broth for mutant selection. *L. crispatus* K313 was routinely cultivated in de Man, Rogosa and Sharpe (MRS) broth at 37 °C without shaking. *Salmonella* was grown in Luria–Bertani (LB) medium at 37 °C under aeration. All cultures were grown to the stationary growth phase before being used in subsequent experiments.

2.2. Constructing the gshF mutant

The *gshF* gene was mutated by inserting the pG^{+}host9 plasmid containing erythromycin into the active site. The *gshF* mutant was constructed per the previously described method (Baccigalupi, Naclerio, de Felice, & Ricca, 2000). Briefly, *S. thermophilus* ST2017 was cultured overnight in LM17 broth. A 776-bp internal fragment of the *gshF* gene was amplified from this strain's genomic DNA using the primer pair, I*gshF*-F (GAAGATCTATCCTGTCTA GGAGCTCGTAG) and I*gshF*-R (CCGCTCGAGCAGTGTCCATAGTA GTCTGAC), and was subsequently inserted into the temperature-sensitive vector pG^{+}host9 (Biswas, Gruss, Ehrlich, & Maguin, 1993). The resulting plasmid was introduced into *S. thermophilus* ST2017 by electroporation, and the transformants were selected at 30 °C with 5 μg mL^{-1} erythromycin. To obtain the *gshF* mutant, appropriate diluted cultures were plated onto LM17 medium at 42 °C (nonpermissive temperature for plasmid replication) with the selective pressure of erythromycin. Cultures were plated onto the same medium at the same temperature without erythromycin to count the integration rate. The *gshF* was mutated by homologous crossing-over in the genome and verified by PCR amplification with primers T*gshF*-F (CGTGTTTCATTCAGTGATAGA) and T*gshF*-R (TTAAGTTTGACCAGCCACT).

2.3. Determining GSH content

The cellular extracts were prepared, and the GSH content was determined as previously described (Wang et al., 2017).

2.4. Cell survival under oxidative stress

Cell survival under H_2O_2 treatment was assessed as previously described (Thibessard, Leblond-Bourget, Fernandez, Gintz, & Decaris, 2001). Briefly, after overnight static incubation at 42 °C, *S. thermophilus* ST2017 and the *gshF* mutant, were inoculated at 2% into 5 mL fresh LM17 broth and incubated statically at 42 °C. When the OD_{600} reached 0.4, the cell cultures were treated with 15 mM H_2O_2 for 30 min. *S. thermophilus* cell survival under oxidative stress was calculated by determining the numbers of colony-forming units (cfu) per a previous method (Zhang et al., 2015).

2.5. 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, USA) containing 10% (v/v) foetal bovine serum (FBS; Biological Industries, Israel) and 1% (v/v) penicillin-streptomycin solution (100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin: Hyclone, Logan, UT, USA) at 37 °C in a humidified atmosphere under 5% CO_2. After reaching 80% confluence, the cells were detached using 0.25% trypsin-EDTA (Hyclone) and seeded in 6-well culture plates at a density of 8×10^5 cells $well^{-1}$ for the antioxidative assays. After 24 h growth, cells were washed twice with phosphate-buffered saline (PBS; Hyclone), then co-incubated with 1×10^8 cfu mL^{-1} of the *S. thermophilus* strains (OD_{600} 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 controls.

2.6. Cellular tolerance to oxidative stress

HT-29 cell viability under oxidative stress induced by H_2O_2 was determined by the water-soluble tetrazolium-8 (WST-8) assay. Specifically, HT-29 cells were cultured in a 96-well plate at a density of 2×10^4 cells $well^{-1}$ for 24 h. Next, 1×10^8 cfu mL^{-1} of the *S. thermophilus* strains were added to the cells in a serum-free medium. After treatment for 24 h, the cultured media were removed, and the cells were washed twice and treated with 5 mM H_2O_2 in serum-free media for 30 min to induce oxidative stress. Subsequently, the supernatants were removed, and 100 μL $well^{-1}$ of the WST-1 solutions (Dojindo Laboratories Co., Tokyo, Japan) were added to the cells. After incubating for 1 h, the absorbance was measured at 450 nm to evaluate the cell viability.

2.7. Measurement of antioxidant enzyme intracellular activities

For these assays, *S. thermophilus* ST2017 treated cells were washed twice, then incubated in 2 mL of fresh RPMI 1640 medium without FBS containing 2 mM H_2O_2 for 30 min to induce oxidative stress. After treatment, the cells were washed twice and lysed using cell lysis buffer with 1 mM phenylmethylsulfonyl fluoride (PMSF; Beyotime Biotechnology, Beijing, China). The lysates were collected by scraping them from the plates, then centrifuged at 13,000×*g* at 4 °C for 5 min. The supernatants were used immediately to further determine superoxide dismutase (SOD), glutathione peroxidase (GSH-px), and catalase (CAT) activity. The SOD, GSH-px, and CAT activity levels were measured using the corresponding activity assay kits (Nanjing Jiancheng Bioengineering Corporation, Beijing, China) per the manufacturer's instructions. Protein concentration in the supernatant was determined using a Q5000 UV–Vis spectrophotometer at 280 nm.

2.8. Analysis of the gene transcriptions by real-time qPCR

After co-incubating with the bacterial strains, total RNA was extracted from the cells using an RNA Simple total RNA kit (Tiangen, Beijing, China) per the manufacturer's protocols, then reverse transcribed to synthesise the cDNA using a PrimeScript RT reagent kit (TakaRa, Tokyo, Japan) per the manufacturer's instructions. To quantify the gene transcription levels, RT-qPCR was performed with SYBR Premix Ex TaqII (TakaRa) using a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) per the manufacturer's protocols.

Table 1
Real-time qPCR primers used to quantify human mRNAs.

Gene name	Forward (from 5′ to 3′)	Reverse (from 5′ to 3′)
Nrf2	ACACGGTCCACAGCTCATC	TGTCAATCAAATCCATGTCCTG
HO-1	CTTCTTCACCTTCCCCAACA	AGCTCCTGCAACTCCTCAAA
GCLC	GGAGACCAGAGTATGGGAGTT	CCGGCGTTTTCGCATGTTG
β-actin	AGTTGCGTTACACCCTTTCTTG	TCACCTTCACCGTTCCAGTTT

The primer pairs used for the RT-qPCR are shown in Table 1. Relative expression levels for each gene were normalised against β-actin as an internal standard and calculated using the comparative threshold cycle (C_T) method ($2^{-\Delta\Delta C_T}$).

2.9. Enzyme-linked immunosorbent assay of IL-8

To analyse IL-8 secretion, HT-29 cells were seeded into 24-well culture plates at a density of 2×10^5 cells well^{-1} and cultured for 24 h. To determine the *Salmonella*-induced IL-8 secretion, HT-29 cells were treated with *Salmonella* at a multiplicity of infection (MOI) of 100 for 6 h after co-incubating with 1×10^8 cfu mL^{-1} of *S. thermophilus* strains overnight. Following co-incubation, cell culture supernatants were collected from bacteria-treated cells, cell culture particulates were removed by centrifugation, and final samples were frozen at −20 °C until assayed. The IL-8 protein concentration was determined by a sandwich enzyme immune assay using commercially available Human IL-8 ELISA kits (Elabscience, Beijing, China). The assay was performed at room temperature per the manufacturer's instructions using thawed samples.

2.10. Statistical analysis

Experimental data were reported as the means ± SD. Statistical significance between treatment and control conditions was assessed using Student's t-test. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Effect of GSH biosynthesis on defending oxidative stress

To investigate the effect of GSH biosynthesis in defending against oxidative stress, the *gshF* gene was inactivated in the *S. thermophilus* ST2017 genome. As shown in Fig. 1A, the pG$^+$host9 vector was integrated after the homologous arm in the *gshF* gene of the *S. thermophilus* ST2017 genome, and a band of approximately 5000 bp was observed by agarose gel electrophoresis, resulting in the *gshF* mutant. Subsequently, cell survival of the strain ST2017 and the *gshF* mutant generated under oxidative stress were determined, and the results showed that the *gshF* mutant cell survival was three orders of magnitude lower than that of the strain ST2017, while no change was observed between the two strains in the absence of oxidative stress (Fig. 1B). Further study showed that the intracellular GSH content of the *gshF* mutant was approximately 5-fold lower than that of strain ST2017, suggesting that the *gshF* mutant could not effectively synthesise GSH, and GSH biosynthesis by GshF was necessary for *S. thermophilus* to defend against oxidative stress.

3.2. Effect of GSH biosynthesis on cellular tolerance to oxidative stress

To determine the effect of GSH biosynthesis on cellular antioxidant responses, the viability of HT-29 intestinal epithelial cells (IECs) under oxidative stress was evaluated. Fig. 2 shows that when co-incubated with the *gshF* mutant, HT-29 cell viability was less than 10% under H_2O_2 exposure, while strain ST2017 showed no obvious decrease in cell viability under the same condition.

3.3. Effect of GSH biosynthesis on antioxidant enzyme activities of HT-29 cells

To evaluate the effect of GSH biosynthesis by GshF on antioxidant responses in IECs, the activities of three antioxidant enzymes in HT-29 cells were assayed under 2 mM H_2O_2 treatment. First, the H_2O_2 concentration used was determined by preliminary experiments: H_2O_2 concentrations ranging from 10 μM to 5 mM were analysed for the HT-29 cell line, and 2 mM induced maximal oxidative stress while maintaining cell viability at 70% (data not shown). In these experiments, tertiary butylhydroquinone (tBHQ), a synthetic antioxidant, was used as a positive control (Abiko, Miura, Phuc, Shinkai, & Kumagai, 2011). As shown in Fig. 3A, when HT-29 cells were co-incubated with strain ST2017, SOD activity was enhanced by 2-fold under H_2O_2 treatment, while the intracellular SOD activity decreased by 53.7% in *gshF* mutant-treated cells compared with strain ST2017-treated cells.

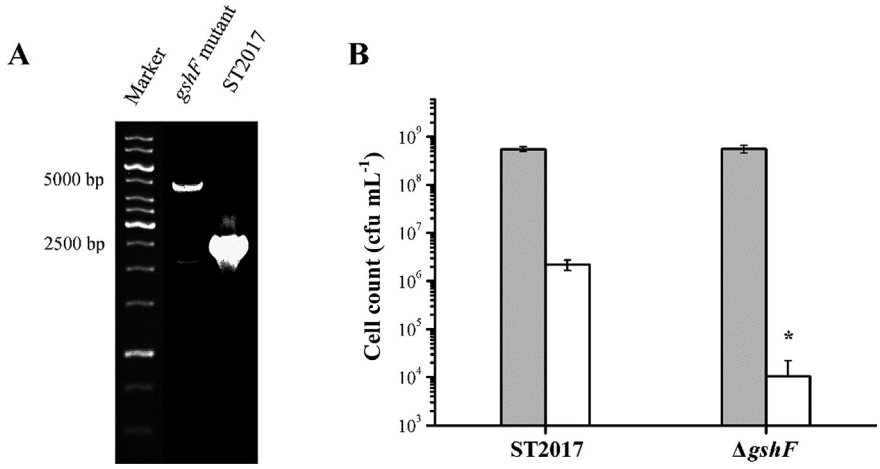

Fig. 1. Verification of *gshF* mutant (A) and cell survival of *S. thermophilus* ST2017 and *gshF* mutant (Δ*gshF*) under normal growth conditions (grey bar) and 15 mM H_2O_2 (white bar) (B). (A) PCR products from *S. thermophilus* ST2017 and its *gshF* mutant. Marker is the GeneRular 1 Kb DNA Ladder, the sizes of the three brightest bands are 6000 bp, 3000 bp and 1000 bp, respectively. In (B), data are shown as the means ± standard deviation, n = 3. An asterisk indicates statistically significant difference compared with strain ST2017 under oxidative stress ($P < 0.01$).

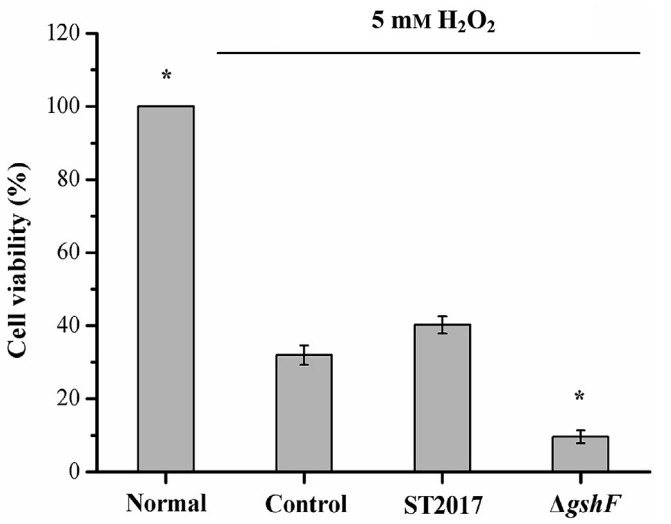

Fig. 2. Effects of *S. thermophilus* ST2017 and the *gshF* mutant (Δ*gshF*) on cellular tolerance to oxidative stress induced by 5 mM H_2O_2. Cell viabilities are presented as percentages relative to the normal group. Normal indicates the untreated cells. Control indicates the cells incubated with H_2O_2 alone. ST2017 and Δ*gshF* indicate the cells incubated with *S. thermophilus* ST2017 and its *gshF* mutant with H_2O_2. Data are shown as the means ± standard deviation, n = 3; an asterisk indicates $P < 0.01$ compared with the control.

The effect of GSH biosynthesis on GSH-px activity in HT-29 cells was also evaluated, and the result is shown in Fig. 3B. Compared with the increased intracellular GSH-px activity of strain ST2017-treated cells, the *gshF* mutant treatment did not affect GSH-px activity under the H_2O_2 condition.

CAT activity in HT-29 cells was also assayed (Fig. 3C). Similar to the SOD and GSH-px activities, the intracellular CAT level decreased to the control level after co-incubation with the *gshF* mutant under oxidative stress.

3.4. Effect of GSH biosynthesis on gene expression of antioxidant enzymes in HT-29 cells

To further investigate the effect of GSH biosynthesis on cellular antioxidant activity, we used RT-qPCR to determine the gene transcription levels of several antioxidant enzymes, including the major antioxidant regulator, nuclear factor erythroid 2-related factor 2 (Nrf2), and Nrf2-related antioxidant enzymes, haeme oxygenase-1 (HO-1) and glutamate-cysteine ligase catalytic subunit (GCLC). Fig. 4 shows that after co-incubating HT-29 cells with *S. thermophilus* ST2017, the transcription levels of *Nrf2*, *HO-1* and *GCLC* in HT-29 cells were elevated to different degrees. However, *gshF* mutant treatment significantly decreased the transcription levels of these genes in HT-29 cells by 2.2-, 4-, and 1.3-fold, respectively.

3.5. Effect of GSH biosynthesis on S. thermophilus anti-inflammatory activity

To investigate the effect of GSH biosynthesis on the inflammatory process in IECs, ELISA was performed to evaluate IL-8 levels. Fig. 5A shows that incubating IECs with *Salmonella* alone increased the IL-8 level from 88.5 pg mL^{-1} in normal cells to 245.9 pg mL^{-1}. When IECs were pre-incubated with strain ST2017, the elevated IL-8 production significantly decreased by 37%–155 pg mL^{-1}, while this attenuation was not observed after *gshF* mutant pretreatment. *Lb. crispatus* K313 was used as the positive control.

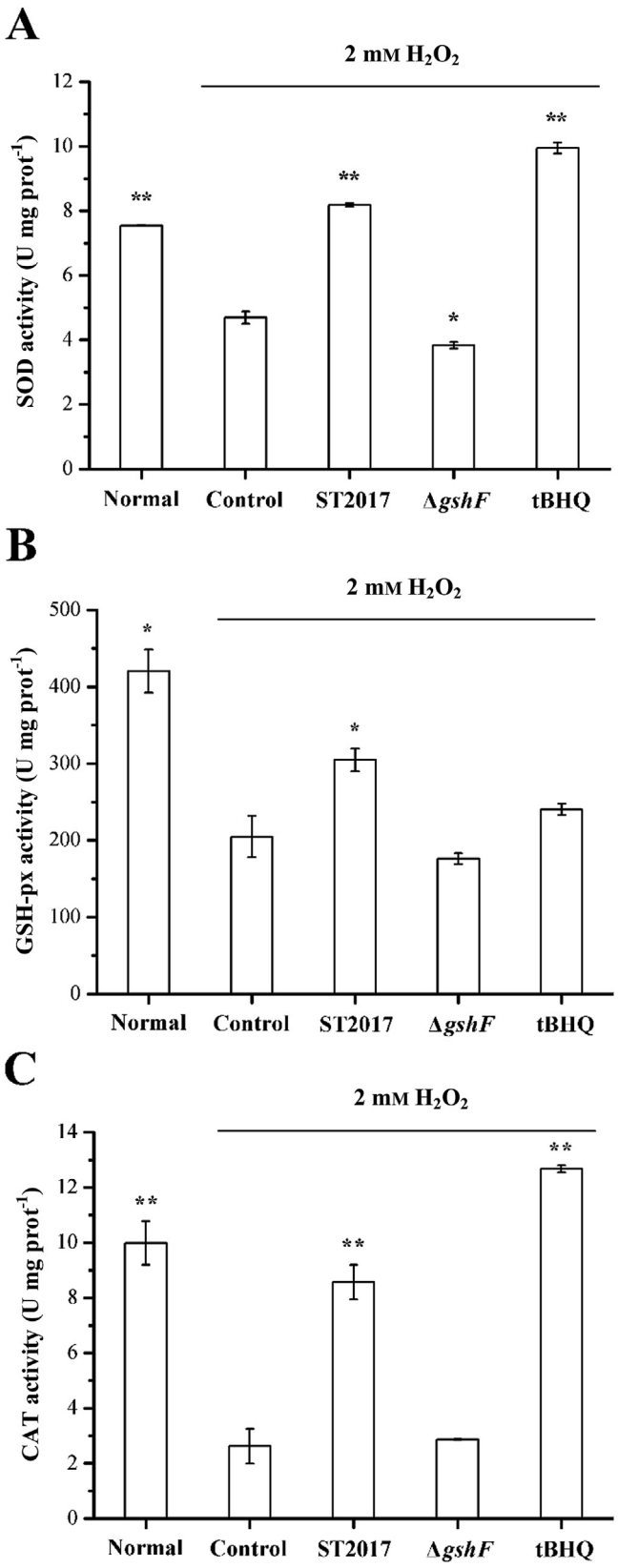

Fig. 3. Effects of *S. thermophilus* ST2017 and *gshF* mutant (Δ*gshF*) on SOD activity (A), GSH-px activity (B) and CAT activity (C) in HT-29 cells. HT-29 cells were co-incubated with strain ST2017, Δ*gshF* and positive control (tBHQ) with H_2O_2, respectively. Values are expressed as means ± standard deviation, n = 3. Normal indicates the untreated cells. Control indicates the cells incubated with H_2O_2 alone. Asterisks indicate statistically significant difference compared with the control group: *, $P < 0.05$; **, $P < 0.01$.

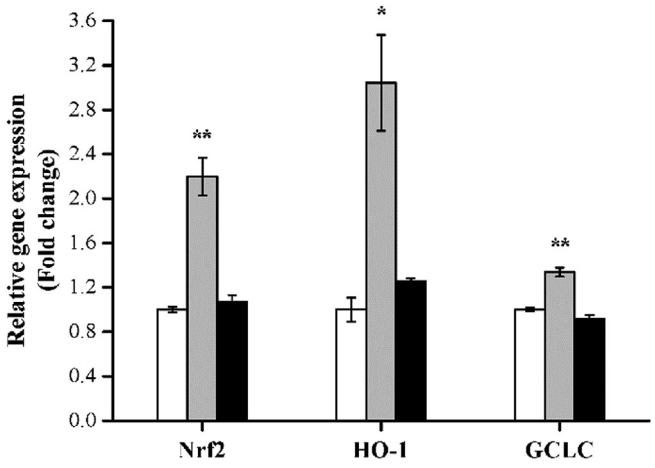

Fig. 4. Effects of *S. thermophilus* ST2017 and *gshF* mutant on gene expression of antioxidant enzymes in HT-29 cells. The gene transcription levels of the antioxidant enzymes, Nrf2, HO-1, and GCLC, were evaluated after co-incubation with strain ST2017 (grey) and *gshF* mutant (black) for 2 h. Relative gene expression levels are presented as the fold change relative to the normal group (white). Data are shown as the means ± standard deviation, n = 3. Asterisks indicate statistically significant difference compared with the normal group: *, $P < 0.05$; **, $P < 0.01$.

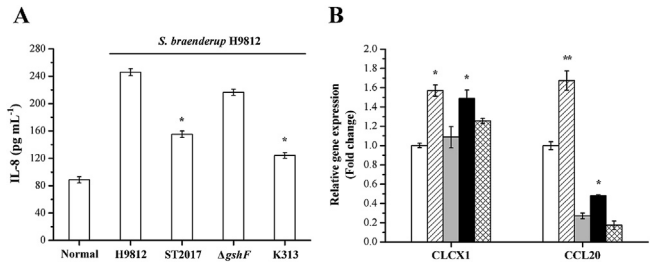

Fig. 5. *S. thermophilus* ST2017 and *gshF* mutant (Δ*gshF*) effects on anti-inflammatory activity induced by *Salmonella braenderup* H9812. IL-8 production (A) was determined after pre-incubating HT-29 cells with strain ST2017 and Δ*gshF* prior to treatment with *Salmonella*. H9812 indicates the cells incubated with *Salmonella* alone, and K313 was used as the positive control. Data are shown as the means ± standard deviation, n = 3. An asterisk indicates $P < 0.05$, compared with the H9812 group. Relative transcription levels of proinflammatory genes, CXCL1 and CCL20 (B), were determined. The striped bar indicates incubation with *Salmonella* alone; the grey, black and grid bars indicate pre-incubation with strain ST2017, Δ*gshF* and K313 prior to treatment with *Salmonella*, respectively. Relative gene expression levels are presented as the fold change relative to the normal group (white). All data are shown as the means ± standard deviation, n = 3. Asterisks indicate statistically significant difference compared with the normal group: *, $P < 0.05$; **, $P < 0.01$).

To further confirm the anti-inflammatory effect, the transcription levels of two key genes involved in proinflammatory responses, CXCL1 and CCL20, were evaluated. Fig. 5B shows that pre-incubation with strain ST2017 significantly reduced the CXCL1 and CCL20 transcription levels induced by *Salmonella* infection by 31% and 83%, respectively. After pre-incubation with the *gshF* mutant, the transcription levels of these two genes were enhanced by 36.7% and 77.8% compared with the strain ST2017 pretreatment, respectively.

4. Discussion

Lactic acid bacteria (LAB) are gaining attention for their potential benefits in treating diseases and preventing inflammation, diarrhoea, cancer and other diseases (Saez-Lara, Gomez-Llorente, Plaza-Diaz, & Gil, 2015; Zhong, Zhang, & Covasa, 2014; Żyżelewicz, Nebesny, Motyl, & Libudzisz, 2010). *S. thermophilus* is a common LAB with several benefits. However, research on its beneficial mechanisms remains limited. Here, we investigated the effect of GSH biosynthesis on *S. thermophilus's* antioxidant and anti-inflammatory activities.

To investigate the effect of GSH biosynthesis on oxidative stress resistance, the *gshF* mutant was constructed by inserting the pG^+host9 plasmid into the *S. thermophilus* ST2017 genome. The resulting mutant could not efficiently synthesise GSH or maintain sensitivity to oxidative stress, suggesting that GSH biosynthesis has important roles in antioxidant activity in *S. thermophilus*. GSH is a cellular bioactive compound possessing many important functions such as antioxidation, acid prevention, and detoxification. Thus, GSH may play a beneficial role by endowing the bacterial strain with good physiological activity. In addition, genomic analysis revealed a putative transport gene, *cydDC*, localised near the *gshF* in the *S. thermophilus* genome (Wang et al., 2015), suggesting that GSH could be transferred out of the cells to exert its activity.

Oxidative stress in the human body occurs from an imbalance between cellular antioxidant capacities and reactive oxygen species (ROS) production (Halliwell, 2009; Żyżelewicz et al., 2016). Excessive ROS and oxidation products cause oxidative damage to cellular biomolecules, such as proteins, nucleic acids, and lipids, impairing their biological functions (Berlett & Stadtman, 1997). As an edible probiotic strain, *S. thermophilus* possesses antioxidant activity for host cells. Due to their endogenous ability to induce ROS production in eukaryotic cell models (Craig & Slauch, 2009; Watterlot et al., 2010), bacteria generally cause oxidative damage or have deleterious effects on cells. Thus, *S. thermophilus* ST2017 did not notably enhance cellular tolerance to oxidative stress. However, the *gshF* mutant markedly decreased HT-29 cell viability, suggesting that inactivating *gshF* rendered the strains unable to properly perform their antioxidant functions and was therefore essential for the beneficial effects of *S. thermophilus*.

SOD, GSH-px, and CAT are key antioxidant enzymes in cells. SOD transforms the superoxide anion into H_2O and O_2 with the assistance of CAT. GSH is an electron donor for glutathione peroxidase, enabling the reduction of H_2O_2 into H_2O, thus preventing cells from damage by H_2O_2 and its derivatives. SOD, GSH-px and CAT activities were evaluated to determine cellular changes in antioxidant status. The tBHQ is a synthetic antioxidant that enhances SOD and CAT's intracellular activities. Our results indicated that co-incubating HT-29 cells with *S. thermophilus* ST2017 significantly promoted SOD and CAT activities in HT-29 cells, although not to the effect of tBHQ, and enhanced the GSH-px activities. However, *gshF* deficiency decreased the activities of these three enzymes to varying degrees, suggesting that GSH biosynthesis by GshF played an important role in *S. thermophilus's* alleviating oxidative stress in IECs. Nrf2 serves as a master regulator of a cellular defence system against oxidative stress, controlling the gene expression of multiple antioxidant enzymes with an antioxidant response element (ARE) in their promoter regions (Jaiswal, 2004). The decreased transcription levels of Nrf2-related antioxidant enzymes following co-incubation with the *gshF* mutant further confirmed the cytoprotective effect of GSH biosynthesis against oxidative stress.

Many diseases are associated with inflammatory responses such as inflammatory bowel diseases (IBDs) and *Salmonella*-induced diarrhoea. *Salmonella* is a common pathogen that causes diarrhoea (Hartnack, van Metre, & Morley, 2012). *Salmonella* infection stimulates the production of cytokines, chemokine IL-8, CXCL1 and CCL20, in IECs (Deligios, 2009). *S. thermophilus* can effectively inhibit pro-inflammatory reactions, thereby reducing the risk of disease. GSH has antioxidant and anti-inflammatory effects, so we hypothesised that GSH biosynthesis played an important role in the anti-inflammatory activity os *S. thermophilus*. As expected, IL-8

ELISA in this study showed that *S. thermophilus* ST2017 exhibited a stronger anti-inflammatory effect on *Salmonella*-infected human IECs than did the *gshF* mutant. Further study suggested that pre-incubation with strain ST2017 could reduce CXCL1 production, while *gshF* mutant pretreatment did not alleviate the increased CXCL1 transcription level induced by the *Salmonella* infection. Although the CCL20 transcription level in the *gshF* mutant-treated cells was significantly lower than that of the control and H9812-treated cells, it was remarkably higher than that of the strain ST2017-treated cells. Overall, these results suggested that GSH biosynthesis in *S. thermophilus* was associated with inhibiting pro-inflammatory responses in IECs.

5. Conclusions

In conclusion, GSH biosynthesis not only protected *S. thermophilus* against oxidative stress, but also played an important role in *S. thermophilus* relieving oxidative stress in IECs. Furthermore, GSH biosynthesis inhibited the pro-inflammatory responses by IECs, thus endowing *S. thermophilus* with its anti-inflammatory effects. Our findings suggest that GSH biosynthesis promotes antioxidant and anti-inflammatory activities and is essential for the beneficial effects of *S. thermophilus*.

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References

Abiko, Y., Miura, T., Phuc, B. H., Shinkai, Y., & Kumagai, Y. (2011). Participation of covalent modification of Keap1 in the activation of Nrf2 by tert-butylbenzoquinone, an electrophilic metabolite of butylated hydroxyanisole. *Toxicology and Applied Pharmacology, 255*, 32–39.

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. *Applied Microbiology and Biotechnology, 97*, 809–817.

Baccigalupi, L., Naclerio, G., de Felice, M., & Ricca, E. (2000). Efficient insertional mutagenesis in *Streptococcus thermophilus*. *Gene, 258*, 9–14.

Berlett, B. S., & Stadtman, E. R. (1997). Protein oxidation in aging, disease, and oxidative stress. *Journal of Biological Chemistry, 272*, 20313–20316.

Biswas, I., Gruss, A., Ehrlich, S. D., & Maguin, E. (1993). High-efficiency gene inactivation and replacement system for gram-positive bacteria. *Journal of Bacteriology, 175*, 3628–3635.

Borgo, F., Carpen, A., Ferrario, C., Iametti, S., & Fortina, M. G. (2013). Genomic analysis reveals the biotechnological ability of *Enterococcus italicus* to produce glutathione. *Journal of Industrial Microbiology and Biotechnology, 40*, 489–494.

Brigidi, P., Swennen, E., Vitali, B., Rossi, M., & Matteuzzi, D. (2003). PCR detection of *Bifidobacterium* strains and *Streptococcus thermophilus* in feces of human subjects after oral bacteriotherapy and yogurt consumption. *International Journal of Food Microbiology, 81*, 203–209.

Copley, S. D., & Dhillon, J. K. (2002). Lateral gene transfer and parallel evolution in the history of glutathione biosynthesis genes. *Genome Biology, 3*, 1–16.

Craig, M., & Slauch, J. M. (2009). Phagocytic superoxide specifically damages an extracytoplasmic target to inhibit or kill *Salmonella*. *PLoS One, 4*, e4975.

Deligios, M. (2009). *Structural and functional genomic analysis of the* Salmonella enterica *host-restricted serotype abortusovis* (PhD Thesis). Sassari, Italy: University of Sassari Department of Biomedical Sciences.

Elli, M., Callegari, M. L., Ferrari, S., Bessi, E., Cattivelli, D., Soldi, S., et al. (2006). Survival of yogurt bacteria in the human gut. *Applied and Environmental Microbiology, 72*, 5113–5117.

Halliwell, B. (2009). The wanderings of a free radical. *Free Radical Biology and Medicine, 46*, 531–542.

Hartnack, A. K., van Metre, D. C., & Morley, P. S. (2012). *Salmonella enterica* shedding in hospitalized horses and associations with diarrhea occurrence among their stablemates and gastrointestinal-related illness or death following discharge. *Journal of the American Veterinary Medical Association, 240*, 726–733.

Hols, P., Hancy, F., Fontaine, L., Grossiord, B., Prozzi, D., Leblond-Bourget, N., et al. (2005). New insights in the molecular biology and physiology of *Streptococcus thermophilus* revealed by comparative genomics. *FEMS Microbiology Reviews, 29*, 435–463.

Jaiswal, A. K. (2004). Nrf2 signaling in coordinated activation of antioxidant gene expression. *Free Radical Biology and Medicine, 36*, 1199–1207.

Junjua, M., Kechaou, N., Chain, F., Awussi, A. A., Roussel, Y., Perrin, C., et al. (2016). A large scale in vitro screening of *Streptococcus thermophilus* strains revealed strains with a high anti-inflammatory potential. *LWT Food Science and Technology, 70*, 78–87.

Kebouchi, M., Galia, W., Genay, M., Soligot, C., Lecomte, X., Awussi, A. A., et al. (2016). Implication of sortase-dependent proteins of *Streptococcus thermophilus* in adhesion to human intestinal epithelial cell lines and bile salt tolerance. *Applied Microbiology and Biotechnology, 100*, 3667–3679.

Li, Y., Hugenholtz, J., Abee, T., & Molenaar, D. (2003). Glutathione protects *Lactococcus lactis* against oxidative stress. *Applied Microbiology and Biotechnology, 69*, 5739–5745.

Li, W., Li, Z., Yang, J., & Ye, Q. (2011). Production of glutathione using a bifunctional enzyme encoded by *gshF* from *Streptococcus thermophilus* expressed in *Escherichia coli*. *Journal of Bacteriology, 154*, 261–268.

Mater, D. D. G., Bretigny, L., Firmesse, O., Flores, M. J., Mogenet, A., Bresson, J. L., et al. (2005). *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* survive gastrointestinal transit of healthy volunteers consuming yogurt. *FEMS Microbiology Letters, 250*, 185–187.

Naidu, A. S., Bidlack, W. R., & Clemens, R. A. (1999). Probiotic spectra of lactic acid bacteria (LAB). *Critical Reviews in Food Science and Nutrition, 39*, 13–126.

Nebesny, E., & Żyżelewicz, D. (2006). Properties of chocolates enriched with viable lactic acid bacteria. *Deutsche Lebensmittel-Rundschau, 102*, 27–32.

Pagnini, C., Saeed, R., Bamias, G., Arseneau, K. O., Pizarro, T. T., & Cominelli, F. (2010). Probiotics promote gut health through stimulation of epithelial innate immunity. *Proceedings of the National Academy of Sciences of the United States of America, 107*, 454–459.

Pophaly, S. D., Singh, R., Pophaly, S. D., Kaushik, J. K., & Tomar, S. K. (2012). Current status and emerging role of glutathione in food grade lactic acid bacteria. *Microbial Cell Factories, 11*, 114.

Rabot, S., Rafter, J., Rijkers, G. T., Watzl, B., & Antoine, J. M. (2010). Guidance for substantiating the evidence for beneficial effects of probiotics: Impact of probiotics on digestive system metabolism. *Journal of Nutrition, 140*, 677S–689S.

Saez-Lara, M. J., Gomez-Llorente, C., Plaza-Diaz, J., & Gil, A. (2015). The role of probiotic lactic acid bacteria and bifidobacteria in the prevention and treatment of inflammatory bowel disease and other related diseases: A systematic review of randomized human clinical trials. *BioMed Research International, 2015*, 505878.

Savaiano, D. A. (2014). Lactose digestion from yogurt: Mechanism and relevance. *American Journal of Clinical Nutrition, 99*, 1251S–1255S.

Sun, Z., Huang, L., Kong, J., Hu, S., Zhang, X., & Kong, W. (2012). In vitro evaluation of *Lactobacillus crispatus* K313 and K243: High-adhesion activity and anti-inflammatory effect on *Salmonella braenderup* infected intestinal epithelial cell. *Veterinary Microbiology, 159*, 212–220.

Thibessard, A., Leblond-Bourget, N., Fernandez, A., Gintz, B., & Decaris, B. (2001). Response of *Streptococcus thermophilus* CNRZ368 and its colonial variants to oxidative stress: Evidence for an inducible defence system. *Le Lait, 81*, 311–316.

Vergauwen, B., de Vos, D., & van Beeumen, J. J. (2006). Characterization of the bifunctional γ-glutamate-cysteine ligase/glutathione synthetase (GshF) of *Pasteurella multocida*. *Journal of Biological Chemistry, 281*, 4380–4394.

Wang, Y., He, H. Y., Li, H. H., Lu, W. W., Guo, T. T., & Kong, J. (2017). The global regulator CodY responds to oxidative stress by the regulation of glutathione biosynthesis in *Streptococcus thermophilus*. *Journal of Dairy Science, 100*, 8768–8775.

Wang, T., Lu, W. W., Lu, S. Y., & Kong, J. (2015). Protective role of glutathione against oxidative stress in *Streptococcus thermophilus*. *International Dairy Journal, 45*, 41–47.

Wang, T., Xu, Z., Lu, S., Xin, M., & Kong, J. (2016). Effects of glutathione on acid stress resistance and symbiosis between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. *International Dairy Journal, 61*, 22–28.

Watterlot, L., Rochat, T., Sokol, H., Cherbuy, C., Bouloufa, I., Lefevre, F., et al. (2010). Intragastric administration of a superoxide dismutase-producing recombinant *Lactobacillus casei* BL23 strain attenuates DSS colitis in mice. *International Journal of Food Microbiology, 144*, 35–41.

Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R., & Turner, N. D. (2004). Glutathione metabolism and its implications for health. *Journal of Nutrition, 134*, 489–492.

Zhang, C. C., Xin, Y. P., Wang, Y., Guo, T. T., Lu, S. Y., & Kong, J. (2015). Identification of a novel dye-decolorizing peroxidase, EfeB, translocated by a twin-arginine translocation system in *Streptococcus thermophilus* CGMCC 7.179. *Applied and Environmental Microbiology, 81*, 6108–6119.

Zhong, L., Zhang, X., & Covasa, M. (2014). Emerging roles of lactic acid bacteria in protection against colorectal cancer. *World Journal of Gastroenterology, 20*, 7878–7886.

Żyżelewicz, D., Nebesny, E., Motyl, I., & Libudzisz, Z. (2010). Effect of milk chocolate supplementation with lyophilized *Lactobacillus* cells on its attributes. *Czech Journal of Food Science, 28*, 392–406.

Żyżelewicz, D., Zakłos-Szyda, M., Juśkiewicz, J., Bojczuk, M., Oracz, J., Budryn, G., et al. (2016). Cocoa bean (*Theobroma cacao* L.) phenolic extracts as PTP1B inhibitors, hepatic HepG2 and pancreatic βTC3 cell cytoprotective agents and their influence on oxidative stress in rats. *Food Research International, 89*, 946–957.