The effect of glutathione biosynthesis of Streptococcus thermophilus ST-1 on cocultured Lactobacillus delbrueckii ssp. bulgaricus ATCC11842

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ABSTRACT

Streptococcus thermophilus and Lactobacillus delbrueckii ssp. bulgaricus are the main species for yogurt preparation. Glutathione (GSH) can be synthesized by S. thermophilus and plays a crucial role in combating environmental stress. However, the effect of GSH biosynthesis by S. thermophilus on cocultured L. delbrueckii ssp. bulgaricus is still unknown. In this study, a mutant S. thermophilus ΔgshF was constructed by deleting the GSH synthase. The wild strain S. thermophilus ST-1 and ΔgshF mutants were cocultured with L. delbrueckii ssp. bulgaricus ATCC11842 by using Transwell chambers (Guangzhou Shuopu Biotechnology Co., Ltd.), respectively. It was proven that the GSH synthesized by S. thermophilus ST-1 could be absorbed and used by L. delbrueckii ssp. bulgaricus ATCC11842, and promote growth ability and stress tolerance of L. delbrueckii ssp. bulgaricus ATCC11842. The biomass of L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ST-1 or ΔgshF (adding exogenous GSH) increased by 1.8 and 1.4 times compared with the biomass of L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ΔgshF. Meanwhile, after H2O2 and low-temperature treatments, the bacterial viability of L. delbrueckii ssp. bulgaricus cocultured with S. thermophilus ΔgshF, with or without GSH, was decreased by 41 and 15% compared with that of L. delbrueckii ssp. bulgaricus cocultured with S. thermophilus ST-1. Furthermore, transcriptome analysis showed that the expression levels of genes involved in purine nucleotide and pyrimidine nucleotide metabolism in L. delbrueckii ssp. bulgaricus ATCC11842 were at least 3 times increased when cocultured with S. thermophilus (fold change > 3.0). Moreover, compared with the mutant strain ΔgshF, the wild-type strain ST-1 could shorten the fermented curd time by 5.3 hours during yogurt preparation. These results indicated that the GSH synthesized by S. thermophilus during cocultivation effectively enhanced the activity of L. delbrueckii ssp. bulgaricus and significantly improved the quality of fermented milk.

Key words: Streptococcus thermophilus, Lactobacillus delbrueckii ssp. bulgaricus, glutathione, cocultivation, yogurt

INTRODUCTION

Streptococcus thermophilus is the second most important bacterium in the dairy industry after Lactococcus lactis and is widely used in the manufacture of various fermented foods due to its acidifying ability (Lecomte et al., 2016). Its physiological, technical, and functional properties in the production of dairy products have been well characterized (Boulay et al., 2020). For example, S. thermophilus can synthesize extracellular polysaccharides. This characteristic contributes to the better viscous and rheological properties of fermented dairy products (Delorme, 2008). Furthermore, S. thermophilus may be able to release a variety of biologically active peptides from β-CN during the fermentation of milk, which has a positive impact on consumer health (Miclo et al., 2012). Lactobacillus delbrueckii ssp. bulgaricus is mainly known for its application in yogurt production worldwide. During the long-term evolution, L. delbrueckii ssp. bulgaricus has adapted to the nutrient-rich milk environment (van de Guchte et al., 2006; Hao et al., 2011). Furthermore, a variety of volatile organic compounds including aldehydes, ketones, and hydrocarbons are produced, resulting in the good flavor of yogurt (Dan et al., 2017a,b). However, the pH of fermented milk gradually decreases with the increase of fermentation time, which impairs the bacterial viability of the starters. Therefore, how to improve the tolerance of L. delbrueckii ssp. bulgaricus against acid environment has become a research focus.
Milk is rich in nutrients such as protein, fat, lactose, and minerals, but is low in free AA. That limits the individual growth of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. To survive better, these 2 species have formed synergistic symbiotic relationships during their use in milk. Experiments have proved that the growth and acid production capacity of the mixed culture is greatly improved compared with single-species culture. During the coculture, *S. thermophilus* absorbs and uses the AA and peptides produced by the proteolytic system of *L. delbrueckii* ssp. *bulgaricus*. Meanwhile, *L. delbrueckii* ssp. *bulgaricus* benefits from the substances such as formic acid, folic acid, pyruvate, carbon dioxide, glutathione (GSH), and long-chain fatty acids produced by *S. thermophilus* (Sieuwerts et al., 2008; Kaneko et al., 2014). Furthermore, studies have shown that both *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* can grow and survive slowly under low H2O2 and weak acid conditions under the cocultivation. It proves that they can regulate their metabolism to enhance their resistance to these environmental stresses (Thibessard et al., 2004, 2011; Li et al., 2015). Therefore, the symbiotic relationship between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* can be reflected by not only the exchange of nutrients, but also the joint resistance to environmental stress (Herve-Jimenez et al., 2009; Sieuwerts et al., 2010).

Glutathione is a tripeptide composed of glutamic acid, cysteine, and glycine. It can be synthesized by the majority of eukaryotes and gram-negative bacteria, and some of the gram-positive bacteria (Li et al., 2003; Pophaly et al., 2012). Glutathione is a crucial nonprotein sulfhydryl compound in these organisms. As a very important component in the antioxidant mechanism of lactic acid bacteria (LAB), it plays an important role in reducing free radicals, maintaining the reducing environment in cells, and improving cell stress resistance, thus protecting cells from oxidative damage and other chemical stressors (Forman et al., 2009; Hatem et al., 2017). Previous studies have shown that *S. thermophilus* can synthesize GSH and transport the GSH out of the cells, although *L. delbrueckii* ssp. *bulgaricus* cannot synthesize GSH by itself (Wang et al., 2015, 2016). Therefore, the effect of GSH biosynthesis of *S. thermophilus* on cocultured *L. delbrueckii* ssp. *bulgaricus* is worth studying. In this study, a GSH synthetase (GSHF) mutant (*S. thermophilus* Δ*gshF*) was constructed. Then the effect of GSH biosynthesis in *S. thermophilus* on the growth and stress resistance of *L. delbrueckii* ssp. *bulgaricus* were investigated by using Transwell chambers (Guangzhou Shuopu Biotechnology Co., Ltd.). Moreover, yogurt fermentation was conducted to determine the effect of GSH synthesis in *S. thermophilus* on the cocultured species in the yogurt environment.

**MATERIALS AND METHODS**

**Strains, Medium, and Culture Conditions**

No animals were used in this study, and ethical approval for the use of animals was thus deemed unnecessary. The wild-type strain *S. thermophilus* ST-1 and its mutant were grown at 42°C in LM17 medium (Thermo Fisher Oxoid) according to the research method of Wang et al. (2016). For transformants selection, 10 mg/mL of erythromycin was added to the LM17 medium. When required, 0.5 mM GSH (Sangon Bioengineering Co., Ltd.) was supplemented into the LM17 medium. *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC11842 was cultured statically at 37°C in de Man, Rogosa, and Sharpe (MRS) medium (Thermo Fisher Oxoid). Chemically defined media were prepared as previously described with modification (Letort and Juillard, 2001). The medium consisted of 10 g of glucose, 5 g of sodium acetate, 3 g of potassium dihydrogen phosphate, 3 g of dipotassium hydrogen phosphate, 0.2 g of magnesium sulfate heptahydrate, 0.05 g of manganese sulfate heptahydrate, 0.02 g of ferrous sulfate heptahydrate, 0.02 g of ferrous sulfate heptahydrate, 0.1 g of alanine, 0.1 g of arginine, 0.2 g of aspartic acid, 0.2 g of cysteine, 0.2 g of glutamine, 0.1 g of glycine, 0.1 g of histidine, 0.1 g of isoleucine, 0.1 g of leucine, 0.1 g of lysine, 0.1 g of phenylalanine acid, 0.1 g of methionine, 0.1 g of proline, 0.1 g of serine, 0.1 g of threonine, 0.1 g of tryptophan, 0.1 g of tyrosine, 0.1 g of valine, 0.001 g of hydrochloric acid VB3, 0.001 g of pantothenic acid VB5, 0.002 g of pyridoxal VB6, 0.001 g of riboflavin VB2, 0.01 g of p-amino benzoic acid, 0.001 g of folic acid, 0.001 g of cyanocobalamin VB12, 0.01 g of D-biotin, 0.01 g of thiamine, 0.01 g of uracil, and 0.01 g of thymine, which was dissolved in 1 L of distilled water. The pH value of the chemically defined medium was adjusted to 6.8. After sterilization by filtration, it was stored at 4°C until further use. All chemicals were reagent grade.

**Construction of Mutant S. thermophilus ΔgshF**

A stable mutant of *S. thermophilus* deficient in GSH synthase was constructed by 2 successive homologous recombination steps (Gilberti et al., 2002; Palumbo et al., 2004). According to the whole gene sequence of *S. thermophilus* deposited in the GenBank database (www.ncbi.nlm.nih.gov/nuccore/NZ_LR822015.1; accession number: NZ_CP038020), characteristic primers Up-F/R and Down-F/R were designed. The
genomic DNA of *S. thermophilus* ST-1 was extracted by using FastPure Bacteria DNA Isolation Mini Kit (Vazyme Biotech Co., Ltd.). Then, primer sets Up-F (5′-GGCGCTCGAGAAGACAAAAATTGGTAAGAG-3′)/Up-R (5′-CATTAAGTAGAAGGTTGATTCCTTAGCTGAACCTTGGAC-3′) and Down-F (5′-AGAATCACCCTTTTCTATCTAAGT-3′)/Down-R (5′-ATAGGATCCCACATCGATGCCATT CATAAAG-3′) were designed to amplify the upstream and downstream of target gene *gshF*. Subsequently, these 2 fragments were ligated by overlapping splicing PCR. Finally, the upstream and downstream gene segments of the gene *gshF* was inserted into the plasmid pG+host9 by the method of homologous recombination (ClonExpress II, Vazyme Biotech Co., Ltd.) to obtain the plasmid pG+host9-up-down. The resulting plasmid was transformed into *S. thermophilus* ST-1 by electroporation as follows. Overnight cultures of *S. thermophilus* ST-1 in LM17 were inoculated into 5 mL of LM17 at a ratio of 2%. An equal volume of pretreatment buffer (M17 broth with 0.5% sucrose, 20% glycerol, and 0.8 M sorbitol) was added to the cultures when *S. thermophilus* ST-1 grew to the optical density at 600 nm of 0.5. After another 1 h of incubation at 42°C and 30 min in an ice bath, the cells were harvested and washed in the ice-cold electroporation buffer (0.1 M sorbitol, 10% glycerol) by alternate centrifugation and resuspension. At last, the cells were resuspended in 50 μL of electroporation buffer. For electroporation, the prepared competent cells and 0.5 μL of plasmid DNA were electro-roporated in a 0.2-cm ice-cold cuvette at 12.50 kV/cm by using an electroporator (Eppendorf 2510). Then, the recovery medium (M17 broth with 0.5% sucrose, 20% glycerol, and 0.8 M CaCl2) was added and incubated at 30°C for 2 h. The transformants were selected with 2.5 μg/mL of erythromycin at 30°C. Subsequently, the obtained strain with single crossing over was inoculated into LM17 medium containing erythromycin and cultured at 42°C. The Δ*gshF* mutant was constructed by knocking out the target gene by double crossing over. Polymerase chain reaction verification was carried out by using primers Up-F and Down-R. Sequencing was then performed to ensure the correctness of the mutant strains.

### Determination of GSH Content

*Streptococcus thermophilus* was cultured to the stationary phase and collected by centrifugation (10,000 × g, 10 min). The cells were washed twice with 0.85% sodium chloride solution, and then resuspended with 1 mL of phosphate buffer B (0.2 M Na2HPO4, 0.2 M NaH2PO4, 2 mM EDTA, pH 7.0, stored at 4°C after sterilization). We added 0.1 g of glass beads into 500 μL of the bacterial solution. The mixture was shaken 3 times (6,000 × g, 30 s, 4°C) by a Precellys24 homogenizer (Bertin). The cell debris was discarded by centrifugation (12,000 × g, 10 min, 4°C), and the supernatant was collected to detect the GSH content. The 35 μL of 0.3 mM NADPH, 10 μL of GSH sample, and 5 μL of 6 mM 5,5-dithiobis (2-nitrobenzoic acid; DTNB) was made up to a 50 μL reaction system and equilibrated at 25°C. Subsequently, 1 μL of 50 U/mL of GSH reductase (Sangon Biotechnology Co., Ltd.) from the baker’s yeast was added to the pre-warmed solution and transferred to a cuvette with a 1-cm light path. Finally, the samples were continuously monitored for changes in absorbance at a wavelength of 412 nm using a spectrophotometer (UV-5500PC; Shanghai Metash Instruments Co., Ltd.). We prepared NADPH, DTNB, GSH, and GSH reductase all in a phosphate buffer (0.125 M K2HPO4, 6.3 mM EDTA, pH 7.5).

### Establishment of Coculture Model by Transwell Chamber

The Transwell chambers were used to assess the growth of *L. delbrueckii* ssp. *bulgaricus* ATCC11842 under conditions of coculture with *S. thermophilus* strains. The *S. thermophilus* ST-1 or Δ*gshF* was inoculated into the upper chamber, and *L. delbrueckii* ssp. *bulgaricus* ATCC11842 was cultured in the lower chamber. Between these 2 chambers, Anopore® filter membrane with a size of 0.22 μm was used to separate the strains and exchange the medium composition. *Streptococcus thermophilus* ST-1 and Δ*gshF* were pre-cultured overnight at 42°C in LM17 medium, and then transferred to theupper chamber of the Transwell containing LM17 medium, respectively. Because the biomass of *S. thermophilus* ST-1 is significantly higher than that of *S. thermophilus* Δ*gshF*, the biomass of *S. thermophilus* ST-1 and Δ*gshF* was adjusted to the same concentration with 0.85% NaCl solution before further inoculum in the following experiments. *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC11842 was pre-cultured at 37°C overnight in chemically defined medium, and then transferred to the lower chamber of the Transwell containing MRS medium. Then, the Transwell chamber was incubated at 42°C.

### Environmental Stress Treatment

*Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC11842 cells of the exponential stage under the coculture model were collected by centrifugation at 6,000 × g for 3 min. After washing twice with 0.85% NaCl solution, the cells were resuspended with 0.85% NaCl solution and adjusted to the same optical density value. The oxidative
stress was performed by addition and treatment with 10 mM H₂O₂ for 30 min. For low-temperature treatment, the suspension was stored at 4°C for 5 d. After culturing L. delbrueckii ssp. bulgaricus to exponential phase, the cells were collected by centrifugation at 3,000 × g and 25°C for 3 min and washed twice with PBS buffer (pH 7.4). The above bacterial cells were subjected to acid stress treatment with a PBS buffer solution of pH 3.5 (the PBS buffer of pH 7.4 was adjusted to pH 3.5 with lactic acid) for 30 min. Then, the lactic acid on the surface was washed with PBS buffer (pH 7.4).

After these stress treatments, the bacterial solution was diluted to 10⁻¹⁻¹⁰ with 0.85% NaCl solution, and the 5 μL of diluted solution was seeded on MRS plate, respectively. The bacterial count of L. delbrueckii ssp. bulgaricus cocultured with the wild-type strain of S. thermophilus ST-1 was used as a control. The colonies were counted to determine and calculate the survival rate of L. delbrueckii ssp. bulgaricus ATCC11842.

Transcriptome Analysis

For transcriptome analysis, we refer to parts of the methods of Kanehisa et al. (2004) and Radakovits et al. (2012). The RNA was extracted from L. delbrueckii ssp. bulgaricus ATCC11842 by RNA extraction kit (Takara Bio). The rRNA was removed by rRNA removal kit (Takara Bio). The quantity and purity of RNA were detected by RNA-specific agarose gel electrophoresis and the Agilent 2100 Bioanalyzer (Agilent) and then was broken into 200 to 300 bp fragments by ion breaking. The first strand of cDNA was synthesized by using random primers and reverse transcriptase. When synthesizing the second strand of cDNA, a strand-specific library was established, and deoxynthymidine triphosphate (dTTP) is replaced by deoxyuridine triphosphate (dUTP) to improve the accuracy. The cDNA fragments with a size of 300 to 400 bp were selected according to the results of agarose gel electrophoresis to enrich the library fragments. In the quality control step, the Agilent 2100 Bioanalyzer real-time PCR system was used to measure the library size, and the total concentration of the library was measured by fluorescence quantification. The sequencing was performed on the Illumina platform. Finally, the differentially expressed genes were analyzed, including Gene Ontology enrichment, Kyoto Encyclopedia of Genes and Genomes enrichment, and differential gene aggregation.

Preparation of Fermented Milk

Streptococcus thermophilus ST-1 was cultured overnight at 42°C in LM17 medium, and L. delbrueckii ssp. bulgaricus ATCC11842 was cultured overnight at 37°C in MRS medium. The fermented milk was prepared by inoculation 2% (vol/vol) S. thermophilus and 1% L. delbrueckii ssp. bulgaricus ATCC11842 to milk supplemented with 6% (wt/vol) sucrose. The fermentation was conducted in a 42°C incubator until the milk curdled. Finally, low-temperature after-cooking is carried out for 8 to 10 h, and refrigerated at 4°C, and various indicators of the fermented milk were detected.

The lactic acid degree of the fermented milk was measured using a titration method as follows. We diluted 10 g of fermented milk with 20 mL of double-distilled H₂O in a conical flask. Then, 2 to 3 drops of 0.5% phenolphthalein alcohol solution was added. Finally, 0.1 mol/L of NaOH standard solution was added to the mixture until the color turned red and did not disappear within 1 min. The acidity (“T”) was calculated as the volume of used NaOH standard solution multiplied by 10. The viscosity of fermented milk was measured by using a DV-1 viscometer (Shangrao Hongxiang Industrial Co. Ltd.) with the parameters of 60 rpm, torque 10 to 100%, and time 30 s. Meanwhile, 1 mL of fermented milk was diluted to 10⁻⁸ with 0.85% NaCl solution. Then, 5 μL of the diluted samples were spread on LM17 plates. After anaerobic incubation at 42°C for 24 h, the number of viable bacteria was counted.

Statistical Analysis

Each experiment was repeated at least 3 times. We used SPSS 22.0 software for statistical analysis. The results were expressed as mean ± standard deviation. A P-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Construction of Mutant S. thermophilus ΔgshF

To study the effect of GSH synthesized by S. thermophilus ST-1 on L. delbrueckii ssp. bulgaricus ATCC11842, a mutant ΔgshF that cannot synthesize GSH was constructed by knocking out the gene gshF encoding GSHF in the genome of S. thermophilus ST-1. The homology arm of the gene gshF was integrated into the temperature-sensitive plasmid pGhost9, and then introduced into S. thermophilus ST-1 to inactivate the gene gshF. As shown in Figure 1A, a 2,000 bp band was appeared in the agarose gel electrophoresis, indicating the S. thermophilus ΔgshF was successfully constructed. Subsequently, the intracellular GSH content of S. thermophilus ST-1 and S. thermophilus ΔgshF with and without exogenous GSH supplementation was determined. As shown in Figure 1B, the
intracellular GSH content of *S. thermophilus ΔgshF* was almost undetectable, whereas the intracellular GSH content of *S. thermophilus* ST-1 strain was 6.1 μg/mg of total protein of *S. thermophilus* (*prot*). Meanwhile, it was observed that both strains could effectively absorb exogenous GSH. In the medium supplemented with 0.5 mM GSH, the intracellular GSH content of *S. thermophilus* ST-1 and Δ*gshF* was 7.4 μg/mg prot and 2.8 μg/mg prot, respectively. These results indicated that *S. thermophilus ΔgshF* was unable to synthesize GSH by itself, but still could effectively absorb exogenously supplemented GSH for utilization. Furthermore, the complementing plasmid pMG36e-gshF was also constructed and transformed into *S. thermophilus ΔgshF* to obtain the complementing strain *S. thermophilus ΔgshF*/pMG36e-gshF. Results showed that the complementing strain exhibited a little weak growth tendency compared with the wild strain *S. thermophilus* ST-1. However, the extracellular GSH between these 2 strains was similar (Supplemental Figure S1, https://data.mendeley.com/datasets/hcpmhzsz9d/1). Therefore, only the wild-type *S. thermophilus* ST-1 was chosen as the control in the following experiments.

Pophaly et al. (2017) found that many food-grade LAB had bifunctional fusion protein for GSH biosynthesis and other enzymes for GSH metabolism, which indicated the ability to synthesize and use GSH in these bacteria de novo. However, after exploration and verification, it was found that *S. thermophilus* was the only species that could produce GSH among these LAB. The study by Allam et al. (2018) also found that GSH secreted by *S. thermophilus* had good cadmium binding ability and antioxidant properties. Many studies have shown that GSH can improve the ability of *S. thermophilus* to resist acid stress, oxygen stress, and osmotic stress (Wang et al., 2016; Qiao et al., 2018). However, there are few reports on the role of the GSH synthesis in *S. thermophilus* on its growth and reproduction. In this study, the GSH synthesis-related gene *gshF* in *S. thermophilus* ST-1 was knocked out by the temperature-sensitive plasmid pGhost9. This markerless gene knockout method could avoid the adverse effect of antibiotics on the growth of *S. thermophilus* ST-1. By comparing the intracellular GSH content of *S. thermophilus* ST-1 (2.1 μg/mg prot) with GSH and *S. thermophilus ΔgshF* (0 μg/mg prot) without GSH, it was found that *S. thermophilus ΔgshF* could absorb the exogenous GSH. However, the exogenous GSH could not restore the intracellular GSH content of *S. thermophilus ΔgshF* to the level of wild-type *S. thermophilus* ST-1. Therefore, it is speculated that GSH production by GSH synthase was much more efficient than GSH absorption in *S. thermophilus*.

![Figure 1](https://data.mendeley.com/datasets/hcpmhzsz9d/1)

Figure 1. (A) The PCR verification of the gene knockout of *gshF*. Lane 1 is a DNA marker with a size of 2,000 bp. Lanes 2 and 3 are PCR validation of *Streptococcus thermophilus* ST-1 and *S. thermophilus ΔgshF*, respectively. (B) The intracellular glutathione (GSH) content of *S. thermophilus* ST-1 and *S. thermophilus ΔgshF* cultured with or without exogenous GSH, respectively. ** indicates an extremely significant difference in GSH content compared with the *S. thermophilus* ST-1 group (P < 0.01). (C) Growth curves of *S. thermophilus* ST-1 and *S. thermophilus ΔgshF* without exogenous addition of GSH in chemically defined medium. Error bars represent standard errors from 3 replicate experiments. These values were expressed as the mean ± standard deviation of 3 separate experiments (n = 3). Prot-1 = total protein of *S. thermophilus*; OD = optical density.
Effects of GSH Synthesized by *S. thermophilus* on the Growth of *L. delbrueckii* ssp. *bulgaricus*

The effect of GSH synthesized by *S. thermophilus* on *L. delbrueckii* ssp. *bulgaricus* was investigated by using a Transwell coculture model. To better elucidate the interaction between the 2 species, a 0.22-μm Anopore™ filter was used to separate the 2 species and exchange the nutrients. Based on the coculture model, the growth curve, and the number of viable bacteria, the intracellular GSH content of *L. delbrueckii* ssp. *bulgaricus* ATCC11842 was determined. As shown in Figure 2A, the biomass of *L. delbrueckii* ssp. *bulgaricus* cocultured with *S. thermophilus* ST-1 or ΔgshF (adding exogenous GSH) increased by 1.8 and 1.4 times compared with the biomass of *L. delbrueckii* ssp. *bulgaricus* cocultured with *S. thermophilus* ΔgshF. Furthermore, *L. delbrueckii* ssp. *bulgaricus* ATCC11842 cocultured with *S. thermophilus* ST-1 also had the highest number of viable bacteria. The results indicated that GSH synthesized by *S. thermophilus* ST-1 could significantly promote the growth and survival of *L. delbrueckii* ssp. *bulgaricus* ATCC11842. In different coculture systems, it was found that the intracellular GSH content of *L. delbrueckii* ssp. *bulgaricus* ATCC11842 was almost undetectable when cocultured with *S. thermophilus* ΔgshF. When exogenously GSH was added, a higher GSH content was detected in *L. delbrueckii* ssp. *bulgaricus* ATCC11842, but it was still far below the intracellular GSH content of *L. delbrueckii* ssp. *bulgaricus* ATCC11842, which cocultured with *S. thermophilus* ST-1 (without exogenous GSH).

*Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus* maintain a symbiotic relationship through the exchange of nutrients, such as AA, formic acid, and carbon dioxide (Herve-Jimenez et al., 2009). Previous studies have shown that *L. delbrueckii* ssp. *bulgaricus* cannot synthesize GSH de novo, but can take up GSH from the medium. *S. thermophilus* can transport self-synthesized GSH into the medium and promote the growth of *L. delbrueckii* ssp. *bulgaricus* (Pophaly et al., 2012; Wang et al., 2016; Xu et al., 2021). Furthermore, GSH plays a crucial role in protecting strains from oxidative damage and maintaining redox homeostasis (Forman et al., 2009). The different effects of exogenous GSH and *S. thermophilus*-derived GSH on *L. delbrueckii* ssp. *bulgaricus* are still unknown. In this experiment, *L. delbrueckii* ssp. *bulgaricus* ATCC11842 was cocultured with *S. thermophilus* ST-1 and ΔgshF, respectively. By measuring the growth curve, viable count, and GSH content of *L. delbrueckii* ssp. *bulgaricus* ATCC11842, it was found that GSH synthesized by *S. thermophilus* could be better absorbed by *L. delbrueckii* ssp. *bulgaricus* ATCC11842 compared with exogenously added

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**Figure 2.** The (A) growth curve, (B) viable count, and (C) intracellular glutathione (GSH) content of *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC11842 when cocultured with *Streptococcus thermophilus* ST-1, *S. thermophilus* ΔgshF+GSH, and *S. thermophilus* ΔgshF with the addition of exogenous GSH. Error bars represent standard errors from 3 replicate experiments. The values are expressed as the mean ± standard deviation of 3 separate experiments (n = 3). * indicates a significant difference in viable count or GSH content compared with the *S. thermophilus* ST-1 group (P < 0.05); ** indicates an extremely significant difference in viable count or GSH content compared with the *S. thermophilus* ST-1 group (P < 0.01). OD = optical density; prot-1 = total protein of *S. thermophilus*. 
GSH during coculture, thus playing a crucial role in promoting the growth and survival of L. delbrueckii ssp. bulgaricus ATCC11842. This might be because that the mutation of the gshF gene led to the weakening of other metabolic functions in S. thermophilus, which in turn affected the activities of S. thermophilus and the cocultured L. delbrueckii ssp. bulgaricus. Therefore, the L. delbrueckii ssp. bulgaricus ATCC11842 could not effectively absorb the exogenous GSH when cocultured with S. thermophilus ΔgshF. The underlying mechanism of this phenomenon still needs further study. Nevertheless, it could be concluded that the GSH synthesis pathway in S. thermophilus plays an important role in the symbiotic relationship between L. delbrueckii ssp. bulgaricus and S. thermophilus according to the above results.

**Effects of GSH Synthesized by S. thermophilus on the Resistance of L. delbrueckii ssp. bulgaricus**

To evaluate the effect of GSH synthesized by S. thermophilus ST-1 on the resistance of L. delbrueckii ssp. bulgaricus ATCC11842 to various stresses, the survival of L. delbrueckii ssp. bulgaricus ATCC11842 in the cocultivation model under treatment of 10 mM H2O2, pH 3.5, and 4°C, respectively. The untreated L. delbrueckii ssp. bulgaricus ATCC11842 was used as a control. As shown in Figure 3, when L. delbrueckii ssp. bulgaricus ATCC11842 was cocultured with S. thermophilus ST-1, its viability changed little after these stress treatments. However, the bacterial viability of L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ΔgshF was significantly decreased. In detail, the bacterial viability decreased by 41, 62, and 15% after treatment by 10 mM H2O2 (45 min), pH 3.5 (15 h), and 4°C (15 d), respectively. The bacterial viability of L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ΔgshF and exogenous GSH also significantly decreased. Its viability reduced by 22, 29, and 7.5% after treatment by 10 mM H2O2 (45 min), pH 3.5 (15 h), and 4°C (15 d), respectively. These results indicated that the deletion of gene gshF made S. thermophilus ST-1 unable to synthesize GSH, and L. delbrueckii ssp. bulgaricus ATCC11842 could not absorb GSH produced by S. thermophilus ST-1, thus decreasing the resistance of L. delbrueckii ssp. bulgaricus ATCC11842 to various environmental stress.

Although GSH is rarely found in gram-positive bacteria such as LAB, studies have shown that supplementation with GSH can reduce the effects of various environmental stressors on these species (Surya et al., 2018). As mentioned in previous studies, GSH could improve the resistance of S. thermophilus and L. delbrueckii ssp. bulgaricus to acid stress (Zhang et al., 2018).
Figure 4. Volcano plot comparing gene expression differences of Lactobacillus delbrueckii ssp. bulgaricus ATCC11842 when cocultured with Streptococcus thermophilus ST-1, S. thermophilus ΔgshF, and S. thermophilus ΔgshF with glutathione (GSH). CX1 = L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ST-1; CX3 = L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ΔgshF with GSH; CX6 = L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ΔgshF; down = treatment downregulates the number of expressed genes compared with control; NoDiff = non-significant differentially expressed genes compared with control; up = treatment upregulates the number of genes expressed compared with control.
Log2 fold change = fold relationship of genes differentially expressed among 3 coculture systems.

Table 1. Gene expression difference analysis result statistics

| Control | Treat | Upregulated | Downregulated | Total |
|---------|-------|-------------|---------------|-------|
| CX6     | CX3   | 16          | 4             | 20    |
| CX1     | CX3   | 12          | 10            | 22    |
| CX1     | CX6   | 27          | 27            | 54    |

1 CX6 = Lactobacillus delbrueckii ssp. bulgaricus ATCC11842 cocultured with Streptococcus thermophilus ΔgshF; CX1 = L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ST.

2 CX3 = L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ΔgshF with glutathione.

3 Upregulated genes = treatment upregulates the number of genes expressed compared with control.

4 Downregulated genes = treatment downregulates the number of genes expressed compared with control.

5 Total differentially expressed genes = treatment differentially expressed genes compared with control.

2010b; Wang et al., 2016; Qiao et al., 2018). Streptococcus thermophilus also could improve its resistance to H2O2 by regulating the synthesis of GSH (Wang et al., 2017). At the same time, Zhang et al. (2010a,b) also found that GSH could reduce the effect of low-temperature environment on Lactobacillus sanfranciscensis SK11. In this experiment, we established the exogenous addition of GSH as an experimental group to investigate the effects of GSH produced by S. thermophilus on L. delbrueckii ssp. bulgaricus during cocultivation. It was found that the number of viable bacteria of L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ΔgshF was much lower than that of L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ST-1. These results indicated that GSH could be absorbed by L. delbrueckii ssp. bulgaricus, and played an important role in resistance to environmental stresses. Interestingly, the viable count of L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ΔgshF and exogenous GSH was much lower than that of L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ST-1, which probably resulted from the change in the metabolic function of S. thermophilus ΔgshF.

Transcriptome Analysis of L. delbrueckii ssp. bulgaricus Under Coculture Model

The transcriptome of L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ST-1 (CX1), L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ΔgshF and GSH (CX3), and L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ΔgshF (CX6) was determined, respectively. The volcano plot showed that a total of 96 genes were differentially expressed in the 3 samples. Among them, 20 genes were differentially expressed between CX3 and CX6, 22 genes were differentially expressed between CX3 and CX1, and 54 genes were differentially expressed between CX6 and CX1 (Figure 4 and Table 1).

Transcriptome analysis found that 27 genes were upregulated in CX1 compared with CX6, in which 7 genes were related to the purine nucleotide metabolism pathway. For example, phosphoribosylaminimidazole-azolesuccinocarboxamide synthase-related genes and phosphoribosylformylglycinamidine synthase II-related genes were significantly upregulated (P < 0.05, fold change > 3.0). A total of 16 genes were upregulated in CX3, of which 7 genes were also related to the purine nucleotide metabolism pathway. However, the change fold of L. delbrueckii ssp. bulgaricus ATCC11842 genes in CX3 was lower than that in CX1. Lactobacillus delbrueckii ssp. bulgaricus ATCC11842 in CX1 upregulated 27 genes and 10 genes compared with CX6 and CX3, respectively, of which 4 repeat genes were related

Table 2. Expression levels and differential folds of some genes in Lactobacillus delbrueckii ssp. bulgaricus ATCC11842 in different coculture systems

| Related gene | CX1 | CX3 | CX6 | Log2 fold change |
|--------------|-----|-----|-----|-----------------|
| Phosphoribosylaminimidazole-azolesuccinocarboxamide synthase | 4100.239883 | 2460.770339 | 277.2910933 | 3.0 |
| Phosphoribosylformylglycinamidine synthase II | 6095.428381 | 4687.372211 | 625.9438651 | 4.0 |
| Orotidine 5-apos-phosphate decarboxylase | 2780.904322 | 118.578092 | 111.1203278 | 4.0 |
| Orotate phosphoribosyltransferase | 1607.725638 | 81.7779949 | 87.6729192 | 4.0 |
| Phosphoketolase | 4014.899962 | 9937.048605 | 10715.46574 | 1.0 |
| Glucose-1-phosphate thymidylyltransferase | 1785.271911 | 4290.278057 | 4540.641653 | 1.0 |
| β-galactosidase | 27928.7153 | 56815.26195 | 59564.57354 | 1.0 |
| Lactose permease | 4211.083688 | 9082.468558 | 8820.303454 | 1.0 |

1 Related gene = differentially expressed genes of L. delbrueckii ssp. bulgaricus ATCC11842 in different coculture systems.

2 CX1 = L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with Streptococcus thermophilus ST-1.

3 CX3 = L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ΔgshF and GSH.

4 CX6 = L. delbrueckii ssp. bulgaricus ATCC11842 cocultured with S. thermophilus ΔgshF.

5 Log2 fold change = fold relationship of genes differentially expressed among 3 coculture systems.
to the biosynthesis of pyrimidine nucleotides. For example, the expression of orotidine 5-apos-phosphate decarboxylase-related genes and orotate phosphoribosyltransferase-related genes were significantly upregulated \((P < 0.05, \text{fold change} > 4.0)\). Compared with CX3 and CX6, we detected 8 repetitive genes in CX1, including phosphoketolase, glucose-1-phosphate thymidylyltransferase, β-galactosidase, lactose permease, and other genes related to lactose utilization \((P < 0.05, \text{fold change} > 1.0; \text{Table 2})\).

Previous studies have found that \textit{S. thermophilus} can provide \textit{L. delbrueckii} ssp. \textit{bulgaricus} with substances such as formic acid and folic acid during the cocultivation, and \textit{L. delbrueckii} ssp. \textit{bulgaricus} provides substances such as polypeptides and AA to \textit{S. thermophilus}. The nutrient exchange enables these 2 species to form a symbiotic pattern (Sieuwerts et al., 2010). Based on transcriptome analysis, the synthesis of purine nucleotides in \textit{L. delbrueckii} ssp. \textit{bulgaricus} ATCC11842 was promoted when cocultured with \textit{S. thermophilus} ST-1 or \textit{S. thermophilus} \(\Delta gshF\) with GSH. Furthermore, the beneficial effect of GSH synthesized by \textit{S. thermophilus} ST-1 or \textit{S. thermophilus} \(\Delta gshF\) group decreased significantly. The above experimental results show that GSH synthesized by \textit{S. thermophilus} \(\Delta gshF\)+GSH group and the \textit{S. thermophilus} \(\Delta gshF\) group decreased significantly. As shown in Figure 5A, compared with the \textit{S. thermophilus} \(\Delta gshF\)+GSH group and the \textit{S. thermophilus} \(\Delta gshF\) group, the protein content of fermented milk was decreased significantly. As shown in Figure 5B, the viable counts of \textit{L. delbrueckii} ssp. \textit{bulgaricus} ATCC11842 in the \textit{S. thermophilus} ST-1+GSH group with \(5.95\) h, whereas the longest curd time was obtained by the \textit{S. thermophilus} \(\Delta gshF\) group with \(11.75\) h, and the curd time of the \textit{S. thermophilus} ST-1 group was significantly longer than that of the \textit{S. thermophilus} \(\Delta gshF\)+GSH group and the \textit{S. thermophilus} \(\Delta gshF\) group.

\textbf{Effect of GSH Biosynthesis by \textit{S. thermophilus} on Fermented Milk}

To detect the effect of biosynthetic GSH on the fermentation process and quality of fermented milk, \textit{L. delbrueckii} ssp. \textit{bulgaricus} ATCC11842 with \textit{S. thermophilus} ST-1 or \textit{S. thermophilus} \(\Delta gshF\) were used as starter strains for milk fermentation. The coagulation time, viscosity, acidity, protein content, fat content, and viable bacteria number of fermented milk were detected. As shown in Table 3, the shortest curd time was observed in the \textit{S. thermophilus} ST-1+GSH group with \(5.95\) h, whereas the longest curd time was obtained by the \textit{S. thermophilus} \(\Delta gshF\) group with \(11.75\) h, and the curd time of the \textit{S. thermophilus} ST-1 group was significantly longer than that of the \textit{S. thermophilus} \(\Delta gshF\)+GSH group and the \textit{S. thermophilus} \(\Delta gshF\) group. However, the viscosity and acidity of fermented milk did not change significantly. As shown in Figure 5A, compared with the \textit{S. thermophilus} \(\Delta gshF\)+GSH group and the \textit{S. thermophilus} \(\Delta gshF\) group, the protein content of fermented milk in the \textit{S. thermophilus} ST-1+GSH group and \textit{S. thermophilus} ST-1 group was significantly higher \((P < 0.05)\). As shown in Figure 5B, the viable counts of \textit{L. delbrueckii} ssp. \textit{bulgaricus} ATCC11842 in the \textit{S. thermophilus} ST-1+GSH group and the \textit{S. thermophilus} ST-1 group did not change significantly after 21 d of low-temperature storage. However, the viable counts of \textit{L. delbrueckii} ssp. \textit{bulgaricus} ATCC11842 in the \textit{S. thermophilus} \(\Delta gshF\)+GSH group and \textit{S. thermophilus} \(\Delta gshF\) group decreased significantly. The above experimental results show that GSH synthesized by \textit{S. thermophilus} ST-1 could shorten the curdling time, increase the protein content, and protect the activity of \textit{L. delbrueckii} ssp. \textit{bulgaricus} in the production of fermented milk.

The beneficial active flora contained in fermented milk is, to a certain extent, the basis for the nutritional and health care functions of fermented milk (Parvez et al., 2006; Aryana and Olson, 2017). However, the study by Hamann and Marth (1984) demonstrated that the viable bacteria number of \textit{L. delbrueckii} ssp.
bulgaricus in yogurt decreased significantly after long-term low-temperature storage. In the present study, it could be found that GSH synthesized by S. thermophilus ST-1 protected the activity of L. delbrueckii ssp. bulgaricus ATCC11842, increased the protein content, and improved the nutritional and health functions of fermented milk. In terms of the fermentation time of yogurt, the study by Sodini et al. (2002) reported that it has taken 11.4 h to ferment yogurt with a single S. thermophilus ST7, and co-fermentation by S. thermophilus ST7 and L. delbrueckii ssp. bulgaricus LB12 could shorten the curd time by about 46% (Sodini et al., 2002). However, the milk curd time fermented by ΔgshF group was as long as 11.75 h, suggesting that the GSH provided by S. thermophilus was the key for milk fermentation. Interestingly, although the addition of exogenous GSH could also protect the activity of L. delbrueckii ssp. bulgaricus and reduce the fermentation time, the effect was not as obvious as that of GSH synthesized by S. thermophilus. Therefore, it could be concluded that the GSH secreted by S. thermophilus also played an important role in shortening the fermentation time, improving the activity of the starter, and ensuring the quality of the yogurt.

CONCLUSIONS

In this study, we constructed and functionally characterized an S. thermophilus mutant with GSH synthase knockout. The Transwell coculture experiments proved that GSH synthesized by S. thermophilus could not only promote the growth and survival of L. delbrueckii ssp. bulgaricus, but also play an important role in alleviating the resistance of L. delbrueckii ssp. bulgaricus to oxygen stress, acid stress, and low-temperature stress. In addition, GSH synthesized by S. thermophilus in the process of milk fermentation can significantly shorten the curdling time and improve the quality of fermented milk. Furthermore, GSH synthesized by S. thermophilus could effectively improve the resistance of L. delbrueckii ssp. bulgaricus to the low-temperature environment, which provided the theoretic basis for ensuring the preservation of the nutritional value of fermented milk during long-term low-temperature storage.

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