Orally administered *Streptococcus thermophilus* YIT 2001 is a vehicle for the delivery of glutathione, a reactive reduced thiol, to the intestine


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Running headline: *S. thermophilus* as a carrier of GSH

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Abstract

Aims: We aimed to analyze the behavior of cellular glutathione of *Streptococcus thermophilus* strain YIT 2001 (ST-1) in the gastrointestinal environment to understand how orally administered glutathione in ST-1 cells is delivered stably to the intestine in a reactive form, which is essential for its systemic bioavailability against lipid peroxidation.

Methods and Results: Intracellular glutathione was labeled with L-cysteine containing stable isotopes. ST-1 cells from fresh culture or lyophilized powder were treated with simulated gastric and intestinal juices for 60 min each. The release of intracellular glutathione in digestive juices was quantified via LC–MS/MS. Most of cellular glutathione were retained in the gastric environment and released in response to exposure to the gastrointestinal environment. During digestion, the membrane permeability of propidium iodide increased significantly, especially when cells were exposed to cholate, without change in the cell wall state.

Conclusions: ST-1 cells act as vehicles to protect intracellular reactive components, such as glutathione, from digestive stress, and release them in the upper intestine owing to the disruption of membrane integrity induced by bile acid.
Significance and Impact of Study: ST-1 can potentially act as an intestinal delivery system that can maximize the beneficial effects of reactive thiols when administered orally.

Keywords: Glutathione, gastrointestinal digestion, membrane permeability, lactic acid bacteria, delivery vehicle
Introduction

*Streptococcus thermophilus* is a gram-positive bacterium used widely as a culture starter for the fermentation of dairy products. We have previously reported that *S. thermophilus* YIT 2001 (ST-1) exhibits the highest antioxidative activity among 49 strains of lactic acid bacteria owing to its rich content of cytoplasmic antioxidants (Ito *et al.*, 2003; Ito *et al.*, 2015).

A randomized, double-blind, placebo-controlled trial reported that the oral administration of ST-1 improves the level of oxidized low-density lipoprotein in the peripheral blood of healthy adults and those with mild hypercholesterolemia (Ito *et al.*, 2017). These phenomena relied on the content of bacterial intracellular thiols, particularly glutathione (GSH), indicating the key role of thiols in the physiological effects of ST-1 (Kusuhara *et al.*, 2018). However, the underlying mechanism through which the oral administration of ST-1 efficiently provides the beneficial effect of unstable thiols remains unclear.

GSH is a tripeptide consisting of cysteine, glycine, and glutamic acid. It has well-established antioxidative activity owing to the presence of cysteinyl thiol, which plays an important role in preventing impairment of redox regulation and conjugation to xenobiotics. Thus, numerous nutritional supplements and medicines based on GSH are commercially available. The clinical potential of GSH has been evaluated and discussed for over five decades. Previous studies have demonstrated the potential therapeutic effects of
GSH administration at practical doses in patients with various diseases, including metabolic disorders and viral infections such as coronavirus disease 2019 (Honda et al., 2017; Horowitz et al., 2020; Kalamkar et al., 2021).

Conversely, several clinical trials have shown that the oral supplementation of refined GSH yields no beneficial effects. For instance, Allen et al. reported that the daily oral administration of refined GSH at a dose of 1000 mg per day for 4 weeks resulted in no significant change in the level of lipid peroxidation biomarkers in healthy adults (Allen et al., 2011). It is known that the thiol group of GSH is susceptible to stresses of enzymatic and nonenzymatic conversion into its inactive form (Camera et al., 2002). Thus, the instability of GSH and reactive thiol compounds hinders their oral administration. The liposomal formulation of GSH reportedly exhibits enhanced efficacy at lower doses, such as improvement in levels of blood oxidative stress marker after oral administration of liposomal GSH at a dose of 500 mg per day (Sinha et al., 2018). These findings suggest that although the oral administration of GSH is generally beneficial, owing to its strong reactivity profile, the efficacy of GSH strongly depends on the dosage form and physiological condition of the patient. The reactivity leads to a loss of bioavailability due to oxidation and conjugation in the gastrointestinal tract. During oral probiotic consumption, the cells of microorganisms may act as a vehicle to protect cellular ingredients from digestive stresses,
thereby enhancing the efficacy of probiotics. However, to the best of our knowledge, no studies in the literature have shown the behavior of intracellular components in the digestive juices of *S. thermophilus* and other lactic acid bacteria.

We hypothesized that ST-1 cells can improve GSH bioavailability by serving as a vehicle to protect intracellular reactive components from various stresses of the gastrointestinal tract. This study aimed to assess the GSH release profile to understand the potential mechanism of its release from ST-1 cells into the gastrointestinal tract. This was achieved by evaluating the behavior of intracellular GSH and observing the changes in the cellular structure in an *in vitro* simulation of the gastrointestinal environment.

### Materials and Methods

#### Preparation of cells containing stable isotope-labeled GSH

First, 16% (w/w) skim milk was adjusted to pH 4.6 with hydrochloric acid: the acid supernatant was collected after centrifugation (10 000 × *g*, 4°C, 15 min). The supernatant was then adjusted to pH 7.5 with sodium hydroxide. The whey culture medium was prepared by sterilizing the neutralized supernatant at 115°C for 15 min followed by removal of the precipitant. A stable isotope of L-cysteine (98% U-13C3, 98% 15N; Cambridge Isotope Laboratories Inc, MA, USA) was added to the medium at a final concentration of 0.002% (w/w).
ST-1, obtained from the culture collection of the Yakult Central Institute (Tokyo, Japan), was precultured in modified GAM broth (Nissui Seiyaku CO., Tokyo, Japan) containing 1% lactose for 16 h at 37°C. The ST-1 preculture in modified GAM broth was inoculated into whey medium at a concentration of 0.5% (v/v) and then incubated for 16 h at 34°C. The cells were collected via centrifugation, suspended in saline, or lyophilized with stabilizers (10% sodium L-glutamate, 10% trehalose, 1% trisodium citrate, and 1% sodium L-ascorbate). The colony forming unit (cfu) count of ST-1 was determined via culturing on MRS agar plates (Becton, Dickinson and Company, NJ, USA).

In vitro-simulated gastrointestinal tract tolerance test

In vitro-simulated gastrointestinal digestion was performed in accordance with a previously published procedure with minor modifications (Sako, et al., 2011). Simulated gastric juice (SGJ) and simulated intestinal juice (SIJ) were used for simulating gastric and intestinal digestion, respectively. Based on the previously reported pH values of human gastric juice after a meal, SGJ was prepared at pH 4.5, 4.0, and 3.5 (error range of ±0.1 for each pH) (Dressman, et al., 1990; Gardner, et al., 2002).

SGJ at pH 3.5, 4.0, and 4.5 was prepared by mixing simulated gastric fluid with simulated gastric enzyme solution in a 9:1 volume ratio. The simulated gastric fluid containing...
hydrochloric acid, Bacto Proteose Peptone No. 3 (5.6 g L\(^{-1}\); Thermo Fisher Scientific, MA, USA), sodium chloride (5.6 g L\(^{-1}\)), and sodium hydrogen carbonate (3.3 g L\(^{-1}\)) with porcine gastric mucin (1.7 g L\(^{-1}\); Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) was autoclaved at 115°C for 15 min. The simulated gastric enzyme solution containing pepsin from porcine stomach mucosa (40 μg L\(^{-1}\); Fujifilm Wako Pure Chemical Corporation) was filtered through a 0.22 μm filter. SIJ was prepared by mixing 1 mL of simulated bile fluid (SBF), 4 mL of simulated intestinal fluid (SIF), and 1 mL of simulated pancreatic fluid (SPF) immediately before use. SBF was prepared by mixing ox gall powder (80 g L\(^{-1}\); Becton, Dickinson and Company) and sodium hydrogen carbonate to adjust the pH to 8.0. SIF was prepared by mixing sodium chloride (5 g L\(^{-1}\)), potassium chloride (1 g L\(^{-1}\)), and sodium hydrogen carbonate (3 g L\(^{-1}\)). SPF was prepared by adding pancrelipase to the suspension (20 g L\(^{-1}\), Fujifilm Wako Pure Chemical Corporation). For sterilization, SBF and SIF were autoclaved at 121°C for 15 min; however, SPF was not sterilized. To measure intracellular GSH content and membrane permeability of ST-1 cells during simulated gastrointestinal digestion, digestive juices with an altered composition were used to avoid GSH contamination by bioreagents. In other words, porcine gastric mucin was removed from SGJ, and SBF was replaced with cholate fluid which was prepared by mixing sodium cholate (0.45 g L\(^{-1}\)) and sodium deoxycholate (0.11 g L\(^{-1}\)).
Gastric digestion was simulated by mixing 0.5 mL of fresh or lyophilized ST-1 suspension with 10 mL of SGJ and then incubating the mixture at 37°C for 60 min. An aliquot (2 mL) of the gastric digested sample was mixed with 6 mL of SIJ and incubated at 37°C for 60 min for intestinal digestion. Various measurements of the gastric and gastrointestinal digested samples were collected. Each treatment was performed in triplicate.

Measurement of the extracellular GSH content

After centrifugation (20 000 ×g, 4°C, 10 min) of gastric and gastrointestinal digested samples, 50 μL of the supernatant was added to 10 μL of 100 mg mL$^{-1}$ tris(2-carboxyethyl)phosphine hydrochloride (TCEP•HCl) solution, and the mixture was incubated for 30 min at room temperature. Each reactant was added to a mixture containing 175 μL of 0.5% (w/v, for gastric digested samples) or 0.9% (w/v, for gastrointestinal digested samples) sulfosalicylic acid solution, 25 μL of water, 500 μL of methanol, and 500 μL of chloroform. The mixture was centrifuged for 5 min at 4600 ×g and 4°C, and 100 μL of the supernatant was added to 10 μL of 40 mmol L$^{-1}$ N-ethylmaleimide (NEM) in 15% (v/v) methanol containing 2 mmol L$^{-1}$ disodium EDTA. After derivatization for 60 min at 40°C, 25 μL of NEM-derivatized GSH (>98% glycine–C$_2$, 96%–99% $^{15}$N; Cambridge Isotope Laboratories) in 5% (v/v) methanol (ca. 1 μg mL$^{-1}$) was mixed with 725 μL of water, and the
mixture was applied to a Sep-Pak tC18 column (Waters Corporation, MA, USA). After washing with 1.6 mL of water, the eluent was collected in 1.6 mL of methanol. The methanol fraction was evaporated using an evaporator at 30°C, redissolved in 250 μL of 10% (v/v) methanol, and filtered through a 0.45 μm filter. The filtrate was diluted 10-fold (for gastric digested samples) or 2.5-fold (for gastrointestinal digested samples) with water and transferred into a glass vial for LC–MS/MS as described below. For correcting the extracellular GSH content, marginal recovery rates were obtained for both SGJ and the mixture of SGJ and SIJ (98% ± 3% and 46% ± 3%, respectively), as determined via addition recovery tests, which were performed in triplicate.

Liquid chromatography measurements

An ultrahigh-performance liquid chromatography (UHPLC) system (ACQUITY UPLC, Waters Corporation) equipped with an ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm, Waters Corporation) was used for separation.

For the separation of 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F, Dojindo Laboratories, Kumamoto, Japan)-derivatized intracellular GSH, the column heater and autosampler were maintained at 40°C and 4°C, respectively. A binary mobile phase comprising 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B)
was isocratically (88% A) eluted at a flow rate of 200 μL min\(^{-1}\).

The chromatographic conditions for NEM-derivatized extracellular GSH separation were in accordance with the method of New and Chen (2008) as outlined below. The mobile phase comprised 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B) at a flow rate of 450 μL min\(^{-1}\). A concave gradient from 99.9% A to 98.0% A over 2 min followed by a convex gradient to 70.0% A over 1.5 min was performed. The column was washed with 5.0% A for 1.3 min and returned to the initial condition (i.e. 99.9% A) within a total run time of 6.0 min. The column heater and autosampler were maintained at 60°C and 4°C, respectively.

Mass spectrometry measurements

The eluent from the UHPLC system was directly introduced into the tandem quadrupole mass spectrometer (Xevo TQ-MS, Waters Corporation) in the positive electrospray ionization mode. The \(m/z\) transitions (precursor ion to product ion) for multiple reaction monitoring of ABD-F derivatives were from 505.05 to 376.04, 508.05 to 379.04, and 509.05 to 380.04 for unlabeled, glycine-labeled, and cysteine-labeled GSH, respectively. The \(m/z\) transitions for multiple reaction monitoring of NEM derivatives were from 436.13 to 201.28 and 437.13 to 204.28 for glycine-labeled and cysteine-labeled GSH, respectively. The cone
voltage and collision energy were optimized at 32 and 17 V as well as 24 and 23 V for targeting ABD-F and NEM derivatives, respectively. The capillary voltage was 0.50 kV. The desolvation N$_2$ gas was introduced at a flow rate of 800 L h$^{-1}$ at 500°C. The cone gas flow rate was set at 50 L h$^{-1}$, and the temperature of the ion source was set at 150°C.

Measurement of the intracellular GSH content

Lyophilized cells were washed twice with 10 mmol L$^{-1}$ EDTA and centrifuged for 10 min at 20 000 $\times g$ and 4°C. The precipitate was suspended in 800 μL of 100 mmol L$^{-1}$ borate–potassium chloride buffer (pH 8.0) containing 2 mmol L$^{-1}$ EDTA and then homogenized with 1000 mg of glass beads using FastPrep-24 (MP Biomedicals, CA) for 60 s at 6.5 m s$^{-1}$. The homogenate was centrifuged at 20 000 $\times g$ for 10 min, and 200 μL of the supernatant was derivatized with 300 μL of 1 mmol L$^{-1}$ ABD-F in borate–potassium buffer at 60°C for 15 min.

After the reaction, the solution was cooled on ice for 10 min, and 200 μL of 0.1 mol L$^{-1}$ hydrochloric acid was added. The probed sample was passed through a 0.45 μm filter, and 10 μL of the flowthrough was injected into an HPLC system consisting of a Waters 2695 Alliance system (Waters Corporation) equipped with a Mightysil RP-18GP Aqua column (4.6 × 150 mm, 1.8 μm, Kanto Kagaku Co., Tokyo, Japan) and Waters 2475 fluorescence detector (Waters Corporation). The separation was performed under isocratic conditions with 0.1%
trifluoroacetic acid in water and acetonitrile (88:12, v/v). The flow rate was set at 1.0 mL min\(^{-1}\), and the column was maintained at a temperature of 40°C. The excitation and fluorescence wavelengths for detecting ABD-F-derivatized GSH were set at 380 and 510 nm, respectively.

The content of intracellular stable isotope-labeled GSH was measured via LC–MS/MS to distinguish it from unlabeled GSH using the abovementioned procedure with minor modifications. Fresh ST-1 suspension was diluted 5-fold with saline and further diluted 10-fold with preautoclaved (115°C, 15 min) 16% (v/v) skim milk. After the addition of 10 volumes of 10 mmol L\(^{-1}\) disodium EDTA dihydrate solution, 600 μL of the aliquot was poured into a 1.5 mL tube, and the cells were harvested via centrifugation for 10 min at 20 000 × g and 4°C. The residual cells were mixed with 2500 mg of zirconia/silica beads (0.1 mm, Tomy, Tokyo, Japan) and 800 μL of 100 mmol L\(^{-1}\) borate–potassium chloride buffer at 3200 rpm for 300 s using a bead crusher (μT-12, Titec, Saitama, Japan). After centrifugation for 10 min at 20 000 × g and 4°C, the supernatant was derivatized with ABD-F as described previously. An aliquot (100 μL) of the filtrate was mixed with 10 μL of ABD-F-derivatized GSH (>98% glycine–\(^{13}\)C\(_2\), 96%–99% \(^{15}\)N; Cambridge Isotope Laboratories) solution (ca. 0.8 μg mL\(^{-1}\)), and the mixture was transferred into a glass vial for GSH determination via LC–MS/MS, as described previously.
Preparation of lyophilized cells for assessing cell properties

ST-1 cells were precultured in modified GAM broth (Nissui Seiyaku) containing 1% lactose for 16 h at 37°C. The preculture was inoculated into a medium containing 5% protease-hydrolyzed skim milk (20%, w/v), 0.5% yeast extract (Asahi Brewery, Tokyo, Japan), 0.3% potassium dihydrogen phosphate, 0.4% dipotassium hydrogen phosphate, 0.15% sodium acetate trihydrate, 1% ammonium sulfate, 2% lactose, and 0.06% magnesium sulfate; this medium was adjusted to pH 7.2 with sodium hydroxide and then sterilized for 15 min at 115°C. The bacteria were cultured for 16 h at 34°C via stirring and aeration. The cells were collected via centrifugation and then lyophilized with the previously mentioned stabilizers. The change in the conformation of the lyophilized sample was determined following exposure to digestive juices. The cfu count of ST-1 was determined via culturing on MRS agar plate.

Observation of cell walls via fluorescent staining

The cells were collected from digestive juices via centrifugation (15,000 × g, 15 min, 4°C) and washed twice with phosphate-buffered saline (PBS). The cells were suspended in 70% ethanol/PBS and incubated at room temperature for 30 min. The cells were washed thrice with PBS and subjected to fluorescent staining. The cells were incubated with staining dyes.
(wheat germ agglutinin [WGA] conjugate at a final concentration of 100 μg mL⁻¹, Biotium, CA, USA; 4′,6-diamidino-2-phenylindole [DAPI] at a final concentration of 1 μg mL⁻¹, Dojindo Laboratories) at room temperature for 30 min. The stained cells were then washed once with PBS, collected via centrifugation, and resuspended in PBS. The sample was embedded in 50% (w/v) glycerol/PBS. Glass slides were examined using the Leica Q550FW system, and fluorescence images were analyzed using Image-Pro Plus software (Media Cybernetics, MD, USA).

Assessment of cell membrane permeability

The cells were collected from digestive juices via centrifugation (15 000 ×g, 4°C, 15 min). The cells were suspended in PBS and subjected to fluorescent staining. The cells were incubated with staining dyes (propidium iodide [PI] at a final concentration of 1 μg mL⁻¹, Dojindo Laboratories; DAPI at a final concentration of 1 μg mL⁻¹, Dojindo Laboratories) at room temperature for 5 min. The stained cells were mixed with an equal volume of 2.0% (w/v) low-melting-point agarose (Agarose L, Nippon Gene, Tokyo, Japan) and subsequently embedded in mounting medium (Vectashield H-1000, Vector laboratories). Glass slides were examined using the Leica Q550FW system, and fluorescence images were analyzed using Image-Pro Plus software (Media Cybernetics). The stained cells from six randomly selected
fields were enumerated for each condition.

Statistical analysis

Statistical analyses were done by performing Welch's t-test using the statistical software R (v4.3.0) with RStudio (2023.03.1 + 446) with a probability (P) value of 0.05 indicating a significant difference between two different groups. Bonferroni correction was applied for multiple comparison tests.

Preparation of fermented milk

ST-fermented milk was prepared as described previously (Ito et al., 2017). ST-1 was inoculated in skim milk (16% nonfat solids) with 0.002% L-cystine and incubated until the titratable acidity reached 15.5 % (w/w). The fermented milk was stored at 10°C for up to 30 days.

Results

Production of stable isotope-labeled GSH in ST-1 cells

To distinguish ST-1-derived GSH from that originating from bioreagents (porcine gastric mucin and bile salt), ST-1 cells containing stable isotope-labeled GSH were prepared in
whey medium. The isotope-labeled GSH level in fresh ST-1 cells was 2070 ng mL\(^{-1}\), accounting for 87% of the total intracellular GSH content (Figure 1). After applying this labeling ratio to lyophilized ST-1 cells, for which GSH levels were determined via HPLC based on the assumption that the isotope ratio barely varied during freezing, drying, and storage processes, the isotope-labeled GSH level in lyophilized cells was estimated as 239 μg g\(^{-1}\) (Table 1).

In vitro GSH release study

Fresh or lyophilized ST-1 cells were treated with SGJ and SIJ continuously. The viability of ST-1 cells from fresh culture showed different patterns depending on the pH of SGJ. ST-1 cells from fresh culture showed loss of viability after exposure to SGJ at pH 3.4 (Figure 2a).

Figure 2 (b, c) shows the level of labeled GSH in digestive juices as quantified via LC–MS/MS. The levels of labeled GSH released from fresh cells following gastric digestion at pH 3.4, 4.0, and 4.5 was 180, 38, and 40 ng mL\(^{-1}\), respectively, demonstrating that ST-1 cells retained >90% of GSH when exposed to SGJ (Figure 2b). After intestinal digestion, the levels of labeled GSH in the mixture of SGJ and SIJ at the corresponding pH values were 1580, 1492, and 1157 ng mL\(^{-1}\) (Figure 2c). The release rate of labeled GSH was calculated to be 68%, 70%, and 54% following the intestinal digestion of ST-1 cells exposed to SGJ at pH
3.4, 4.0, and 4.5, respectively. The results demonstrated that fresh cells released >50% of the total intracellular GSH content in the intestinal phase, whereas the release rate in the gastric phase was <10%. The test was also performed for freeze-dried ST-1 cells. The lyophilized cells were suspended in saline and then treated with both digestive juices. The behavior of GSH in lyophilized cells in response to simulated gastrointestinal digestion was similar to that in fresh cells (Table 1). Loss of cell viability was observed after exposure to SGJ at pH 3.5. The intracellular GSH release rate was higher in the intestinal phase than in the other. The levels of labeled GSH released from lyophilized cells were 69 μg g⁻¹ after SGJ treatment and 96 μg g⁻¹ after continuous treatment with SGJ and SIJ. The release rates of cellular GSH were approximately 29% and 40% in the gastric and intestinal phase, respectively.

Changes in membrane permeability and intracellular GSH content

We examined the alterations in the surface structure of ST-1 cells. ST-1 cells in a semisynthetic culture medium were lyophilized and subjected to in vitro-simulated gastrointestinal digestion. ST-1 cells treated with SGJ and SIJ were fluorescently stained with PI and DAPI. The dyeability of ST-1 cells with PI increased with each digestion treatment, and most cells showed penetration of PI after continuous treatment with SGJ.
and SIJ (Figure 3). The ratio of the number of PI-stained cells to the total number of cells increased notably after treatment with SIJ (Figure 4a). Treatment of ST-1 cells with each simulated digestive juice reduced the intracellular GSH content (Figure 4b). The intracellular GSH content decreased by 50% following exposure to SGJ, whereas it was below the limit of detection (92.2 ng mL$^{-1}$) after treatment with SIJ. The release of GSH from ST-1 cells was mostly cholate-dependent; treatment of lyophilized ST-1 cells with SIJ alone reduced the intracellular GSH content, whereas ST-1 cells treated with cholate-free SIJ retained about 65% of the intracellular GSH content (Figure S1). Following simulated gastrointestinal digestion, ST-1 cells were stained with WGA–CF®555 conjugate, which binds specifically to $N$-acetylglucosamine in the outer peptidoglycan layer of gram-positive bacteria, and the dyeability of the cell wall remained unchanged across all digestive stages (Figure 5).

Discussion

This study elucidated the behavior of ST-1 cells and intracellular thiol in the gastrointestinal tract. We evaluated the level of GSH, a typical intracellular antioxidant of ST-1, as shown previously (Kusuhara et al., 2018), as it is a marker that describes the movement of intracellular components. The GSH release profile was assessed using ST-1...
cells containing stable isotope-labeled GSH in an *in vitro*-simulated digestion model. Gastric
digestion at pH 3.4 induced loss of viability in fresh ST-1 cells, with only a small amount of
labeled GSH detected in SGJ. The release of labeled GSH into extracellular fluid was mostly
observed in SIJ. In experiments with lyophilized cells, most of the glutathione was retained
intracellularly in the gastric phase but was eliminated after continuous treatment with SGJ
and SIJ. The characteristics of ST-1 cells were observed in each phase of the simulated
gastrointestinal digestion. The permeability of the cell membrane to PI increased, especially
in the intestinal phase, whereas the dyeability of the cell wall by plant-derived lectin
remained unchanged during treatment. Free bile acids have been reported to integrate into
the lipid bilayer and disrupt the membrane function of lactic acid bacteria, leading to an
increase in permeability (Kurdi *et al.*, 2006). Our findings strongly indicate that ST-1
releases intracellular GSH in response to exposure to digestive juices, particularly bile acid,
by compromising cell membrane integrity. The present study suggests that orally
administered ST-1 acts as a vehicle to deliver and efficiently release intracellular reactive
components, such as GSH, in the upper intestinal tract.

An *in vitro*-simulated gastrointestinal digestion model demonstrated that ST-1 cells
retained most of the intracellular GSH after exposure to gastric juices, despite loss of
viability. Under gastric conditions, damage to bacterial cells occurs due to denaturation of
cell membrane proteins and disruption of the ion gradient, i.e. loss of membrane integrity (Okamoto and Kameya, 2018). In contrast, Robben et al. (2018) reported that bacterial cells in a viable, but not culturable, state are metabolically active and could maintain membrane structure. It is possible that ST-1 cells can maintain membrane permeability despite the damage and act as a vehicle to retain intracellular components under gastric conditions. Kusuhara et al. (2018) reported that orally administered lyophilized ST-1 cells with high intracellular GSH content can improve blood antioxidant parameters in hamsters. In the present study, lyophilized ST-1 cells were used to test the reproducibility of the dosage form of health supplement. Compared with fresh cells from bacterial culture, lyophilization induced the release of intracellular labeled GSH in gastric juices to a greater extent. Several studies have shown that freeze–drying damages bacterial cell membranes and increases the solubility of cellular components (Castro et al., 1997; Musatti et al., 2013). As shown in Figure 3, lyophilized cells had higher membrane permeability in the gastric phase than untreated cells, indicating their sensitivity to acidic conditions. This finding is consistent with the phenomenon that the release of intracellular GSH occurs due to changes in membrane permeability when exposed to digestive juices containing cholate. In addition, we confirmed that approximately 50% of the GSH content was released in the intestinal phase, demonstrating that lyophilized cells exhibit similar characteristics as the vehicle carrying
intracellular components in the simulated gastrointestinal environment. Therefore, it is expected that most lyophilized ST-1 cells have available intracellular antioxidants that can be released in the upper intestine following oral administration.

The instability of GSH and reactive thiol compounds has hindered their clinical application, and this limitation should be overcome to improve the bioavailability. In a previous clinical trial, we reported that the daily oral administration of ST-1-fermented milk for 12 weeks improved the level of peripheral blood oxidative stress biomarker. The calculated dose of GSH derived from ST-1 was approximately 70–130 μg per day (Figure S2), which is markedly lower than the doses specified in previous human trials wherein refined GSH (200–1000 mg per day) was orally administered (Witschi et al., 1992; Allen et al., 2011; Richie et al., 2015; Honda et al., 2017). These findings suggest that ST-1 cells act as a carrier to protect the active component from stresses of the gastrointestinal tract, as proposed in this study. Research on the physiological effects of probiotics has been conducted, and lactic acid bacteria have also been studied for clinical use as delivery vectors to secrete a protein of interest (Cano-Garrido et al., 2015). These technologies not only enable the administration of low-stability molecules without invasive methods, such as intravenous or subcutaneous injection, but also improve the efficiency of the treatment, resulting in lower dosage and a reduced need for repeated administration. The present study suggests the role of ST-1 cells
as a protectant to facilitate the efficient uptake of an unstable compound by the host via the intestinal epithelium. In addition, Uchiyama et al. reported the protective effect of gut bacteria-derived GSH derivatives, such as GSH persulfide, against liver injury induced by oxidative stress (Uchiyama et al., 2022). Therefore, ST-1 may contain other highly reactive GSH derivatives. We previously identified GSH as a typical active component of ST-1: moreover, the inhibitory effect of ST-1 against in vitro oxidation of low-density lipoprotein (LDL) was not completely diminished after 1-methyl-2-vinyl-pyridinium trifluoromethane-sulfonate treatment, which suppresses the antioxidative activity of reactive thiols (Kusuhara et al., 2018), indicating the existence of an unidentified antioxidant.

Overall, the present study assessed GSH release using an in vitro-simulated gastrointestinal digestion model and provided a rationale for the mechanism by which orally administered ST-1 cells act as a protective vehicle to deliver intracellular ingredients and improve the blood oxidative stress status. Our results also revealed the potential of lactic acid bacteria ST-1 as a promising tool and drug delivery vehicle that protects highly reactive, unstable intracellular components from digestive stress. However, there are limitations in the physiological effects of ST-1. Because oxidized LDL is associated with the formation of aortic fatty streaks, ST-1 is considered to exert a protective effect against the formation of...
aortic fatty lesions via the inhibition of lipid peroxidation, as demonstrated previously (Ito et al., 2015). However, the GSH level in ST-1 is lower than that in the human plasma (~10 μmol L⁻¹) (McMenamin et al., 2009). Therefore, other cellular components may contribute to the protective effect of ST-1 through an unknown mechanism. In addition, the fate of intracellular components released in the duodenum remains unknown; further, it remains unclear whether these components reach the peripheral tissues via the bloodstream. A previous study confirmed that orally administered GSH can be transported across the intestinal epithelium in an animal model (Kovacs-Nolan et al., 2014). Considering that several studies have shown the physiological effect of GSH administration on the blood antioxidative status as well as systemic inflammation, skin aging, and cognitive function (Horowitz et al., 2020; Arjinpathana et al., 2012; Mandal et al., 2019), ST-1 may exert systemic effects by oral ingestion via efficient uptake of intracellular active components. Further studies are warranted to determine the mechanism underlying the beneficial effects of orally administered ST-1.

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Conflicts of interest

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Data Availability

The data associated with this article will be shared on reasonable request to the
corresponding author.

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inging), and Akimitsu Takagi (Project administration, Supervision, Writing – review &

inging)
Figure 1

Levels of labeled and unlabeled GSH from ST-1 cells cultured with a stable isotope of L-cysteine.
Figure 2

Behavior of intracellular labeled GSH and fresh ST-1 cells during continuous treatment with simulated gastric juice at different pH and simulated intestinal juice: (a) cell viability after exposure to digestive juices at pH 3.4(○), 4.0(△), and 4.5(□); (b) the level of labeled GSH released from ST-1 cells into simulated gastric juice at various pH; and (c) into simulated intestinal juice. Each value is expressed as the mean and standard deviation. n.d.: not detected. SGJ: simulated gastric juice, SIJ: simulated intestinal juice.
Fluorescent images of ST-1 cells stained with propidium iodide (PI) and 4′,6-diamidino-2-phenylindole (DAPI) after continuous exposure to simulated gastric juice and simulated intestinal juice.
The membrane permeability of ST-1 cells assessed using propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) after continuous exposure to simulated gastric juice and simulated intestinal juice: (a) the ratio of PI-stained cells to total cells (stained with DAPI). (b) The change in the intracellular GSH content of lyophilized cells. Values are presented as the mean ± standard deviation, n = 3. Statistical analysis was performed using Welch’s t-test and corrected for multiple comparisons using the Bonferroni method. * indicates a significant difference between two groups (P < 0.05), n.d.: not detected. SGJ: simulated gastric juice, SIJ: simulated intestinal juice.
Figure 5

Fluorescent images of ST-1 cell wall stained with wheat germ agglutinin-conjugate (WGA) and DAPI after continuous exposure to simulated gastric juice and simulated intestinal juice.