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Effect of Glutathione on the Taste and Texture of Type I Sourdough Bread

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ABSTRACT: Type I sourdough fermentations with *Lactobacillus sanfranciscensis* as predominant organism accumulate reduced glutathione through glutathione reductase (GshR) activity of *L. sanfranciscensis*. Reduced glutathione acts as chain terminator for gluten polymerization but is also kokumi-active and may thus enhance bread taste. This study implemented a type I model sourdough fermentations to quantitate glutathione accumulation sourdough, bread dough, and bread and to assess the effect of *L. sanfranciscensis* GshR on bread volume by comparison of *L. sanfranciscensis* and an isogenic strain devoid of GshR. *L. sanfranciscensis* sourdough accumulated the highest amount of reduced glutathione during proofing. Bread produced with the wild type strain had a lower volume when compared to the *gshR* deficient mutant. The accumulation of γ-glutamyl-cysteine was also higher in *L. sanfranciscensis* sourdoughs when compared to doughs fermented with the *gshR* mutant strain. The accumulation of reduced glutathione in *L. sanfranciscensis* bread did not enhance the saltiness of bread.

KEYWORDS: *type I sourdough, glutathione, sensory, texture, kokumi*

INTRODUCTION

The composition and quality of wheat flour constituents affect rheology and gas holding capacity of wheat and the volume and texture of wheat bread.[1,2] Proteins are major constituents of wheat flour; 80−85% of wheat proteins are gluten proteins, which are composed of monomeric gliadins and polymeric glutenins.[1,2] During the kneading process, polymeric gluten proteins form a viscoelastic protein network, which retains gas produced during fermentation and baking.[1,2] Oxidizing and reducing agents influence gluten polymerization through disulfide bonds and thus affect gluten functionality.[2] Ascorbic acid, cysteine, and glutathione link oxidation by molecular oxygen to disulfide bonding in proteins.[3] Ascorbic acid is widely used as a dough improver to mediate oxidation of glutathione by molecular oxygen. Oxidation proceeds through an enzymatic cascade involving ascorbic acid oxidase and glutathione dehydrogenase.[3,4] During dough mixing, ascorbic acid is oxidized to dehydro-ascorbic acid, which in turn is a cofactor for oxidation of glutathione by glutathione dehydrogenase. Glutathione acts as chain terminator for the gluten macropolymer and thus interferes with the functionality of gluten proteins in wheat baking.[2,3,5] Addition of 0.2−0.3 μmol g^{-1} of reduced glutathione to wheat dough reduces bread volume.[3] A reduction of the glutathione content over short-term storage of white wheat flour corresponds to an increased bread volume.[6] In addition to its role for texture and volume of wheat bread, glutathione is also a "kokumi" taste-active compound.[7] Kokumi substances do not have a distinct taste but enhance the mouthfulness and taste complexity through interaction with the signal transduction from taste receptors to the brain.[8,9]

Lactobacillus sanfranciscensis and most other sourdough lactobacilli are not capable of de novo synthesis of glutathione; however, glutathione reductase activity of sourdough lactobacilli increases thiol levels in sourdough.[10] Negative effect of reduced thiols on gluten functionality may be compensated by solubilization of wheat arabinoxylans and formation of microbial exopolysaccharides during fermentation.[11] Moreover, sourdough fermentation increases the taste complexity of bread,[12] but it remains unknown whether glutathione contributes to this effect.[13] The contribution of sourdough to bread quality depends on the technology of sourdough use. Sourdough has been used as a leavening agent for centuries,[14] and the use of sourdough as the sole leavening agent, also termed type I sourdoughs, persists in contemporary artisanal baking.[15,16] The leavening power of type I sourdoughs is achieved through frequent back-sloping, which keeps sourdough microbiota metabolically active throughout the process. Frequent back-slopping of sourdough selects for rapidly growing organisms; *Lactobacillus sanfranciscensis* is the predominant organism in association with yeasts.[17] In current industrial practice, however, type II sourdoughs are used as baking improver in conjunction with baker's yeast as leavening agent.[16,18] Type II sourdough improve bread quality through accumulation of organic acids, taste-active amino acids, and peptides, or exopolysaccharides.[11]

The accumulation of glutamate and kokumi-active dipeptides during fermentation of type II sourdoughs was linked to the increased taste intensity of bread.[12,13] Fermentation conditions in traditional sourdoughs do not support accumulation of peptides to taste-active concentrations; however, short fermentation times that are typical for traditional, type I fermentations may accumulate glutathione through glutathione reductase activity of *L. sanfranciscensis*.[19] This study aimed to quantitate glutathione accumulation by *L. sanfranciscensis* in sourdough and to determine its influence on the taste and the

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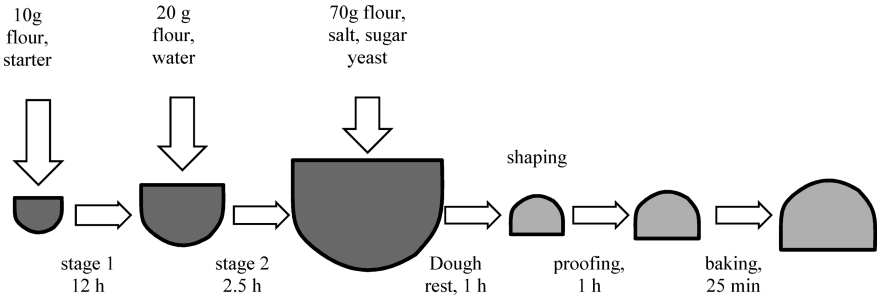

Figure 1. Schematic representation of the sourdough fermentation and baking process. All fermentations were carried out at 32 °C. Stage 1 and stage 2 sourdoughs were fermented with a dough yield of 200; bread dough was mixed with a dough yield of 160. The dough was mixed for 6 min and baked for 25 min at 178 °C.

Table 1. HPLC Parameters Used for Quantitation of Glutathione, γ-Glutamyl-cysteine, and GSSG

	MS	collision energy (CE)	declust potential (DP)	retention time	EXP
glutathione	307→179	20	30	2.66 min	8
γ-glutamyl-cysteine	250.4.3→122.1	22	30	2.43 min	12
GSSG	612.8→355.2	30	30	5 min	8

volume of bread. To specifically assess the role of microbial glutathione reductase on bread taste and volume, the glutathione reductase positive *L. sanfranciscensis* DSM20451^T, which accumulated thiols during sourdough fermentation, was compared to the isogenic but glutathione reductase negative *L. sanfranciscensis* DSM20451^{T}Δ*gshR*. The fermentation protocol used matched procedures that are employed to achieve leavening with sourdough as sole leavening agent. The taste and texture were determined by sensory evaluation, which was performed with untrained consumer panels and trained panels.

MATERIALS AND METHODS

Strains and Growth Conditions. Lactobacilli were cultivated in modified MRS medium (mMRS) containing 10 g L^{-1} of maltose and 5 g L^{-1} of fructose.[20] Additional 10 mg L^{-1} of erythromycin was added to mMRS for cultivating *L. sanfranciscensis* DSM20451^T Δ*gshR*.[19] *L. sanfranciscensis* DSM20451^T, *L. sanfranciscensis* DSM20451^T Δ*gshR*, and *L. sakei* LS8 were cultivated anaerobically at 32 °C. Fermentation inoculum was prepared by harvesting cells from 10 mL of overnight culture at 4000 × *g* for 5 min. Cells were washed twice with sterilized tap water and resuspended to the original volume.

Sourdough Fermentations. The study employed commercial white wheat flour that contained 13% protein, 0.54% ash, and was conditioned with peroxides and ascorbic acid. To prepare the sourdough, 1 g of flour was mixed with 4 mL of cell suspension in water to achieve an initial cell count of 6.94 ± 0.34 log CFU g^{-1}. Sourdoughs were incubated at 32 °C for 24 h. Chemically acidified dough was prepared by adding acetic acid/lactic acid (1:4, v/v) to achieve a dough pH of 3.5 ± 0.25. The concentrations of reduced glutathione (GSH), pH, and cell counts were analyzed immediately after sampling; additional samples were freeze-dried for subsequent analysis by high resolution melt curve analysis quantitative polymerase chain reaction (HRM-qPCR). Fermentations were carried out in triplicate independent experiments, and results were reported the mean ± standard error of the mean.

DNA Extraction from Sourdough and HRM-qPCR. The identity of the fermentation microbiota with the inoculum was confirmed by HRM-qPCR.[21] In brief, DNA was isolated from freeze-dried sourdoughs with the Blood and Tissue Kit (Qiagen, Mississauga, Canada). The DNA concentration was determined by UV spectrometry using a NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA). HRM-qPCR was performed on a Rotor-GeneQ instrument (Qiagen) to determine the melting temperature of 16S rDNA amplicons.[21] PCR reactions were performed with a Type-it HRM PCR kit (Qiagen) and primers 16S−F (AGA6TTTGATYMTGGCTC) and 16S-R (CAKAAAGGA-GGTGATCC) (IDT, Coralville, IA). DNA isolated from overnight cultures of *L. sanfranciscensis* DSM20451^T and *L. sakei* LS8 served as a reference.

Type I Sourdough Bread Baking and Evaluation. A three stage fermentation was implemented to produce type I sourdough bread (Figure 1). Fermentation conditions and the amount of flour added at each fermentation stage largely follow the Detmold three stage sourdough process.[22] In the first stage, 10 g of flour was mixed with 10 mL of inoculum and fermented for 12 h at 32 °C. For the second stage, 20 g of flour and 20 mL of sterilized tap water were added and fermented for 2.5 h. The bread dough was prepared by mixing sourdough, 70 g of flour, 30 mL of sterilized tap water, 2 g of salt, 2 g of sugar, and 0.5 g of yeast (Fleischmann's traditional active dry yeast) for 6 min. After mixing, the dough was rested at 32 °C for 1 h, followed by shaping and proofing at 32 °C for 1 h, and baked at 177 °C for 25 min. Straight dough control bread without sourdough addition was prepared by mixing 100 g of flour with 2 g of salt, 2 g of sugar, and 0.5 g of yeast for 6 min; the remaining protocol was identical to the production of sourdough bread. Bread loafs were cooled down to room temperature for 2 h, packed, and stored at room temperature overnight. Weight and volume of bread were measured 24 h after baking. Loaf volume was determined according to the AACC 10−05 rapeseeds displacement test. The specific volume was calculated by dividing volume by loaf weight. Samples were collected in each step and freeze-dry before liquid chromatography−mass spectrometry (LC−MS) or high-performance LC (HPLC) analysis. Fermentations and bread production were carried out in triplicate independent. Results are reported the mean ± standard error of the mean.

Quantitation of Glutathione by LC−MS/MS. Freeze-dried sourdough or bread (0.1 g) was mixed with 0.5 mL of 1 mM EDTA. The supernatant was collected after centrifugation at 4 °C, and solids were re-extracted with 0.5 mL of 0.1% formic acid. Solids were removed by centrifugation at 4 °C, and the supernatant was combined with the first extract for subsequent analysis. Quantitation was performed using a 1200 series HPLC (Agilent Technologies, Palo Alto, CA) connected to a 4000 Q TRAP LC−MS/MS System (MDS SCIEX, Applied Biosystems, Streetsville, ON, Canada). Peptides were separated on a Synergi 2.5 μHydro RP 100A-HPLC column (100 μm, 3.00 mm × 2.5 mm, Phenomenex, Torrance, CA). Mobile phase A consisted of 0.05% formic acid and 0.05% TFA with 50 mm ammonium formate in Milli-Q water. Mobile phase B consisted of 0.05% formic acid and 0.05% TFA in acetonitrile. The sample was eluted at a flow of 0.5 mL min^{-1} with the following gradient: 0−5 min, 99% A; 5−15 min, 0% A; 15−20 min, 99%A. LC−MS/MS analysis

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was performed using atmospheric pressure electrospray ionization in positive mode. After optimizing individually on the protonated precursor ions and the dominant fragments of glutathione (GSH), dimeric oxidized glutathione (GSSG), and γ-glutamyl-cysteine, samples were detected and quantitated using multiple reaction monitoring mode (MRM). The values for optimum ion source parameters were shown in Table 1. Data acquisition was interfaced to a computer workstation running Analyst 1.5 (Applied Biosystems), which also served as the controlling software for LC/UV system. External calibration standards of GSH, GSSH, and γ-glutamyl-cysteine were prepared in 30% (v/v) methanol in 0.1% aqueous formic acid. Calibration curves consisted of six concentration ranges from 5−0.005 g L^{-1}.

Quantitation of Organic Acids by HPLC. Organic acids in the sourdough and bread were analyzed in triplicate by HPLC (1200 series, Agilent Technologies, USA) equipped with an Aminex HPX 87H column (Bio-Rad) as described.[23] Freeze-dried sourdough or bread (0.1 g) was mixed with 0.5 mL of Milli-Q water and heated in water bath at 80 °C for 2 h. Solids were removed by centrifugation, the supernatant was collected, and 7% perchloric acid was added at 1:1 ratio. The mixture was incubated at 4 °C overnight, and the pellet was removed by centrifugation. Supernatant were filtered and used for analysis. Acetate, lactate, and mannitol were used as external standards.

Sensory Evaluation by Consumer Panel. The sensory studies were reviewed for their adherence to ethical guidelines and approved by the research ethics board at the University of Alberta. The frozen bread was thawed overnight at room temperature before sensory evaluation. The bread was cut into 1 cm^3 pieces with bread crust excluded. For each type of bread, three pieces of crumb were put into 100 mL sample cup labeled with designated three-digit code. Sensory evaluation was performed in the sensory testing laboratory at the Department of Agricultural, Food and Nutritional Science, University of Alberta.

The panels were recruited randomly in the Agricultural and Forestry Centre of the University of Alberta, and all participants expressed written informed consent. The panelists consisted of 36 subjects, 12 male and 24 female; most panelists (69%) were aged between 18 and 29 years. The majority of the panelists (83%) consumed bread more than 2−3 times per week, and about 64% of the panelist's perceived taste is the most important attribute compared to flavor, texture, and color.

The panelists were provided a glass of water, prerandomized bread crumb samples, serviette, pencil, and questionnaire. Panelists were asked to sip water for palate cleansing in between sampling. The samples were presented to the assessors blind so that the panelists did not know which sample they were evaluating. A series of tests was used to compare sensory attributes among *L. sakei* and *L. sanfranciscensis* fermented sourdough bread and regular bread: (1) duo−trio for examining overall difference between *L. sakei* and *L. sanfranciscensis* fermented sourdough breads; (2) paired comparison test on overall difference, saltiness, softness, and sourness between *L. sanfranciscensis* and *L. sakei* sourdough breads; (3) evaluating bread saltiness and softness on Just About Right scale; the verbal anchor for Just About Right scale is from "not at all" to "too much"; and (4) ranking test for saltiness and softness among all three types of bread.

Trained Panel Sensory and Texture Evaluation. Texture evaluations (manual evaluation by compression) for straight dough bread, *L. sanfranciscensis*, *L. sanfranciscensis* Δ*gshR*, and *L. sakei* sourdough bread were performed by trained panelists. Descriptive analysis was used to determine the sensory profiles of the bread samples. Bread that has various distinct attributes was purchased from the local market and used as reference; during the roundtable training session, judges were asked to describe the attributes of reference bread and rank the attribute intensity among reference bread, and agreement on descriptor and ranking was made by consensus. The selected texture attributes were softness and springiness. The softness of bread crumb was evaluated by compressing the center of breadcrumb with a middle finger to about half of its original height; springiness of bread crumb was evaluated based on the speed and degree of the deformed crumb returns to its original shape. Bread loafs were sliced into 1 cm thickness, and end slices of each loaf were discarded. Each slice was placed into a plastic bag with designated three-digit code. Prior to the evaluation, panelists were trained twice on determining bread softness and springiness to ensure the result variances among panelists were minimized. Softness and springiness were evaluated by directional paired comparison tests between *L. sanfranciscensis* and its mutant bread. Panelists also ranked the softness and springiness for all four types of bread to ensure the answers from each panelist were reproducible and reliable. Texture evaluation was repeated twice to ensure reproducibility of results among panelists.

Statistical Analysis. For duo−trio test, the number of correct responses was counted and used statistical chart 4 as described to determine if a detectable overall difference exists between *L. sakei* and *L. sanfranciscensis* sourdough breads.[24] The result for paired comparison test was analyzed by *t* test; for Just About Right scale result, the frequency of each category was calculated. For ranking result, the sum of the rank for each sample was calculated, and the result was analyzed by Friedman method. Statistical analysis for organic acids and GSH concentration in sourdoughs was performed by a two-way analysis of variance and an all pairwise multiple comparison procedure with the Holm-Sidak method. Values were considered significantly different at a 5% error level ($P < 0.05$).

RESULT

Quantitation of Glutathione in Sourdough. To determine the influence of fermentation on glutathione concentration in sourdoughs, doughs fermented with *L. sanfranciscensis* and *L. sanfranciscensis* Δ*gshR* were sampled at 0, 1.5, 3.5, 5, and 24 h, and GSH was quantitated by LC−MS/MS. Chemically acidified doughs were prepared at the same time as a control. The cell count for *L. sanfranciscensis* and *L. sanfranciscensis* Δ*gshR* increased from about 10^7 to 10^9 CFU g^{-1} after 24 h of fermentation. Observation of the colony morphology and HRM-qPCR verified that the fermentation microbiota were identical to the inoculum (data now shown). The cell count of chemically acidified dough was below the detection limit. During the fermentation, the changes of pH for *L. sanfranciscensis* and *L. sanfranciscensis* Δ*gshR* were similar; the pH values after 5 h of fermentation were 4.4 ± 0.54 and 4.52 ± 0.15, respectively; the corresponding pH values after 24 h of fermentation were 3.29 ± 0.06 and 3.46 ± 0.04, respectively.

GSH in sourdough was quantitated by LC−MS/MS in MRM mode (Figure 2). The concentration of GSH for sourdoughs fermented with *L. sanfranciscensis* or *L. sanfranciscensis* Δ*gshR* increased during the exponential growth phase ($P < 0.001$) and decreased to initial values after 24 h of fermentation. The glutathione concentration was higher ($P < 0.05$) in sourdoughs when compared to the chemically acidified control (Figure 2); the GSH concentration in sourdough fermented with *L. sanfranciscensis* was higher than in doughs fermented with *L. sanfranciscensis* Δ*gshR* after 1.5 and 3.5 h of fermentation ($P < 0.05$) but not at other time points (Figure 2). Together, data confirm prior observations that the glutathione reductase of *L. sanfranciscensis* contributes to accumulation of thiols and further indicate that glutathione levels in dough are different only in the exponential phase of growth. Exponentially growing lactobacilli and yeasts are characteristic for type I sourdoughs, while type II sourdoughs employ stationary phase or inactivated cultures. Subsequent experiments to determine the influence of glutathione reductase for bread quality were thus based on a protocol matching the traditional use of type I sourdoughs.

Establishment of Type I Sourdough Fermentation Protocol. Different from traditional type I sourdough fermentation in artisanal bakeries, the benchtop type I

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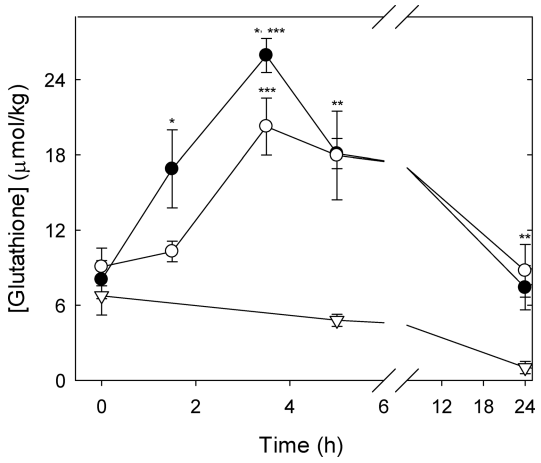

Figure 2. Concentration of glutathione in sourdough fermented with *L. sanfranciscensis* (●), *L. sanfranciscensis* Δ*gshR* (○), and a chemical acidified control (▽). Data are presented as means ± standard error of the mean. Asterisk (∗) indicates statistically significant difference between *L. sanfranciscensis* wild type and *gshR* mutant strains at $P < 0.05$; double asterisks (∗∗) indicate statistical significant difference between sourdoughs and chemically acidified control at $P < 0.05$; triple asterisks (∗∗∗) indicate statistical significant difference between 0 and 3.5 h at $P < 0.001$. ($n = 3$).

sourdough relied on the use of single and defined strains of lactobacilli only in the first two stages of sourdough fermentation. *Saccharomyces cerevisiae* was added to bread dough at 0.5%; this procedure achieved a yeast population in bread dough that qualitatively and quantitatively matches yeasts in traditional type I sourdoughs while maintaining the ability for controlled fermentation with defined strains. Cell counts and pH values at stage I and II of the fermentation are shown in Table 2. The cell counts for all strains were comparable and

Table 2. pH and Cell Counts of Type I Fermented Sourdoughs with *L. sanfranciscensis*, *L. sanfranciscensis* Δ*gshR*, and *L. sakei* at Stages I and II

strain	stage I	stage II
	pH	
L. sanfranciscensis	4.61 ± 0.04	4.77 ± 0.07
L. sanfranciscensis Δ*gshR*	4.05 ± 0.01	4.25 ± 0.05
L. sakei	3.84 ± 0.08	3.89 ± 0.18
	Cell Count Log CFU g^{-1}	
L. sanfranciscensis	8.07 ± 0.15	7.63 ± 0.35
L. sanfranciscensis Δ*gshR*	8.08 ± 0.13	7.21 ± 0.12
L. sakei	8.52 ± 0.11	8.50 ± 0.08

maintained in the stage II fermentation (Table 2); the pH values in sourdoughs fermented with *L. sakei* were consistently lower when compared to sourdoughs fermented with *L. sanfranciscensis* wild type or mutant strains.

Quantitation of Glutathione in a Three Stage Type I Sourdough Fermentation. Concentrations of GSH, γ-glutamyl cysteine and GSSG were quantitated for each of the fermentation steps, after mixing, dough rest, and proofing, and in bread (Figure 3). GSH and γ-glutamyl cysteine changed substantially after dough rest and proofing, which demonstrated that glutathione levels in dough are influenced by enzymatic oxidation, chemical reactions, and microbial reduction or release from lysed cells. *L. sanfranciscensis* sourdough had a significantly higher GSH concentration than sourdough

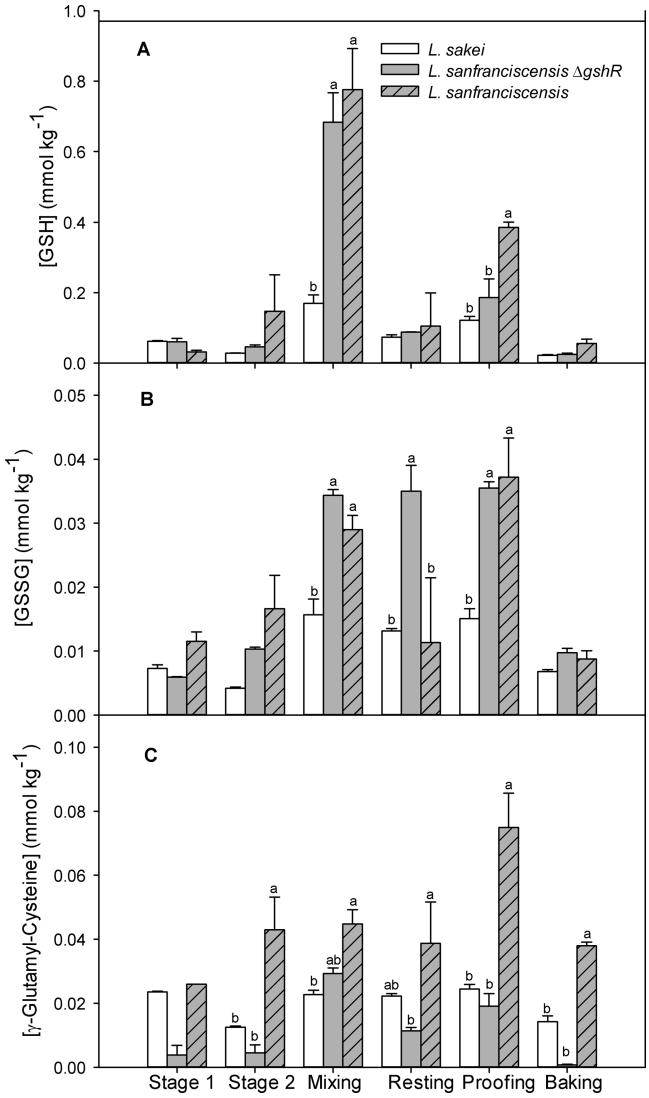

Figure 3. Concentrations of GSH, GSSG, and γ-glutamyl-cysteine in doughs fermented with *L. sakei*, *L. sanfranciscensis* Δ*gshR*, and *L. sanfranciscensis* at different stages. Data are presented as means ± standard error of the mean of triplicate independent fermentations. Values obtained for different sourdoughs at the same stage of fermentation that do not share a common letter differ significantly ($P < 0.05$; $n = 3$).

fermented with *L. sanfranciscensis* Δ*gshR* at proofing, which suggested that GSH levels during mixing and the dough rest are predominantly influenced by enzymatic and chemical reactions during dough rest, while GSH conversion through microbial GshR contributes to GSH levels during proofing. The concentration of γ-glutamyl cysteine also was significantly higher in doughs fermented with the *L. sanfranciscensis* wild type strain after stage 2, resting, proofing, and baking when compared to the corresponding samples fermented with the mutant strain. Because the genome of *L. sanfranciscensis* does not code for a γ-glutamyl cysteine synthetase,[25] the accumulation of the dipeptide γ-glutamyl cysteine reflects enzymatic hydrolysis of GSH. Therefore, quantitation of γ-glutamyl cysteine as a reduced thiol-containing peptide complements the quantitation of GSH. The concentrations of GSSG were low when compared to GSH and high GSH and γ-

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glutamyl cysteine concentrations did not correspond to low GSSG levels (Figure 3).

Sensory Evaluation of Type I Sourdough Bread by Consumer Panel. Sensory evaluation with a consumer panel was employed to assess the effect of glutathione on the taste of bread, and to indicate possible changes in texture. The sensory attributes saltiness, softness, and sourness were evaluated with bread produced with *L. sakei* and *L. sanfranciscensis* sourdoughs or with control bread produced without sourdough. The softness of the three bread was not different (Table 3).

Table 3. Consumer Panel Sensory Result, pH, and Organic Acids (mmol kg^{-1} Dry Matter) Concentration of *L. sanfranciscensis* and *L. sakei* Sourdough Breads and Regular Bread (n = 36)

	L. sanfranciscensis bread	*L. sakei* bread	regular bread
salty taste[1]	68[ab]	75[a]	55[b]
softness	61	69	74
preference	10	12	12
pH	5.42 ± 0.05	5.07 ± 0.17	5.55 ± 0.08
acetate (mmol kg^{-1})	4.13 ± 0.63	2.92 ± 0.00	3.09 ± 0.25
lactate (mmol kg^{-1})	20.4 ± 1.6	27.0 ± 0.32	17.6 ± 2.4

[1]Total sum within a row with different superscript letters are significantly different at $P < 0.05$.

Participants perceived *L. sakei* sourdough bread as "very salty" and "too salty", while control bread and *L. sanfranciscensis* sourdough bread were evaluated as just about right with respect to the salty taste (Figure 4). A second sensory test asked

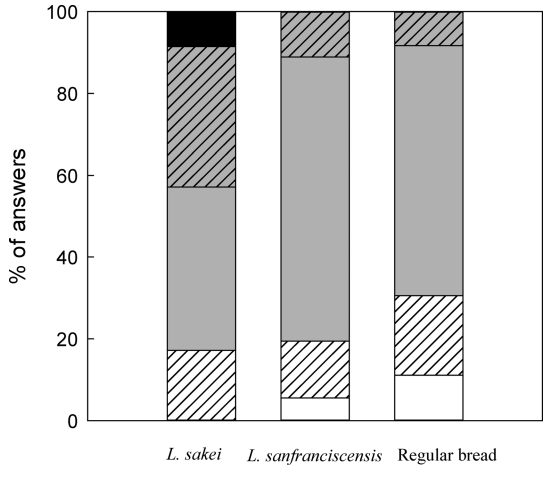

Figure 4. Sensory evaluation of bread and sourdough bread with the "Just about right test" for salty taste and after taste. Sourdough bread was produced with *L. sakei*, *L. sanfranciscensis*, and compared to regular bread obtained with a straight dough fermentation. Bar colors indicate % of answers indicating "not salty at all" (white bars), "little salty" (white hatched bars), "just about right" (gray bars), "very salty" (gray hatched bars), and "too salty" (black bars). n = 35.

consumers to rank bread and sourdough bread with respect to the salty taste intensity (Table 3). This test confirmed that *L. sakei* sourdough bread was saltier than control bread. The consumer panel did not identify significant differences with respect to softness and preference of the breads (Table 3). Differences in pH and organic acids concentrations between the breads were noticeable but not pronounced (Table 3). The acetate concentration was highest in sourdough bread produced with *L. sanfranciscensis*, high acetate concentrations corresponded to high mannitol concentrations, in keeping with the metabolic preferences of heterofermentative lactobacilli (data are not shown).[26] The lactate concentration was highest in bread produced with *L. sakei*, corresponding to a low pH in bread produced with this strain.

Loaf Volume and Texture Evaluation by Trained Panel. To determine whether the role of reduced glutathione in gluten polymerization impacts bread volume and texture, the volume of breads was assessed, and the texture was also evaluated by a trained sensory panel (n = 9). Bread produced with *L. sanfranciscensis* had the lowest specific volume; control bread had an intermediate loaf volume; and bread produced with *L. sakei* or *L. sanfranciscensis* Δ*gshR* sourdough had the highest loaf volume (Figure 5). Bread produced with *L.*

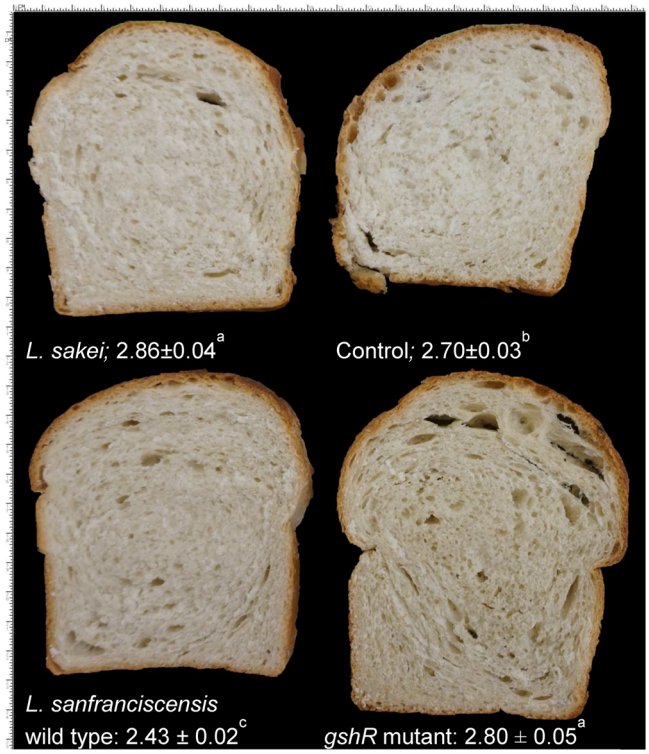

Figure 5. Pictures of bread obtained with type I model sourdough fermentations employing *L. sakei*, *L. sanfranciscensis*, or *L. sanfranciscensis* Δ*gshR*, and control bread obtained with a straight dough process. Numbers indicate the bread volume (cm^3/g) expressed as means ± standard deviation of triplicate independent experiments. Values that do not share a common superscript differ significantly ($P < 0.05$).

sanfranciscensis Δ*gshR* sourdough also had a larger pore size when compared to the wild type strain (Figure 5). Bread softness was evaluated by trained panelists through hand compression method without consumption. Bread produced with *L. sanfranciscensis* Δ*gshR* was softer ($P < 0.05$) when compared to bread produced with the wild type strain in a binary comparison. Bread produced with *L. sanfranciscensis* was also softer ($P < 0.05$) than control bread or bread produced with *L. sakei*, while differences between breads produced with *L. sakei*, *L. sanfranciscensis*, or baker's yeast only were not different ($P > 0.05$).

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DISCUSSION

Development of Type I Model Sourdough. The composition and activity of sourdough microbiota and consequently the influence of sourdough on bread quality depend on the fermentation technology. The use of type I sourdough as a sole leavening agent dictates short fermentation times that maintain fermentation microbiota being continuously metabolically active.[22,26] Type I sourdough fermentation selects for fast growing microorganisms, and fermentation microbiota have been shown to be stable over years or even the decades.[17] *L. sanfranciscensis*, *Candida humilis*, *S. cerevisiae*, and *Saccharomyces exiguus* are the predominant species in type I sourdough.[17,20,26,27] GshR in *L. sanfranciscensis* contributes to oxygen tolerance and supports rapid growth by using GSH as an electron acceptor.[19] We observed that glutathione accumulation in sourdoughs fermented with *L. sanfranciscensis* is only transient (Figure 2). Type I sourdoughs, which are characterized by short fermentation times, thus support accumulation of glutathione, while glutathione is depleted by oxidation or hydrolysis during prolonged type II fermentations. This study therefore employed a laboratory scale type I sourdough fermentation (Figure 1). Different from artisan sourdoughs, laboratory sourdoughs were fermented with single strains of lactobacilli, and yeast was added only at the last fermentation stage. Although yeasts account only for 1−10% of total cell counts in traditional sourdoughs, their metabolic activity generates about half of the CO_2 production.[26] The model sourdough fermentation employed commercial dry yeast to obtain a reproducible inoculum; the cell count of *S. cerevisiae* in the bread dough, 6.6 log CFU g^{-1}, matched the cell counts of *S. cerevisiae* in artisanal fermentation that use sourdough as sole leavening agent.[28] Yeast cell counts in traditional Italian sourdoughs ranged from 6.5−7.6 log CFU g^{-1}, and the predominant yeast species were *S. cerevisiae* and *C. humilis*.[28] The pH of type I sourdough breads ranged from 4 to more than 5;[20,22] bread acidity was adjusted by fine-tuning the relative contribution of yeasts and lactic acid bacteria to metabolic conversions at the dough stage.[22,26] The pH of type I sourdough bread produced in this study at the neutral end of the range that is typical for traditional products,[28] but even at this low acidity level, the accumulation of glutathione affected the bread properties.

Quantitation of Reduced and Oxidized Glutathione in Dough and Bread. Glutathione and GSSG have been quantitated from biological samples with various methods including LC coupled to electrochemical detection or MS.[29,30] GSH quantitation requires prevention of glutathione autoxidation or degradation by proteinases for precise and accurate quantitation.[29,30] In this study, EDTA and temperature control prevented oxidation and enzymatic reactions during sample preparation. Glutathione and GSSG are very hydrophilic so that they are not easily retained on regular reversed-phase columns; the RP 100A column used in this study provided good retention and separation of these two compounds and additionally allowed quantitation of γ-glutamyl cystine, a degradation product of glutathione.

Fate of Glutathione during Sourdough Fermentation. Glutathione dehydrogenase is present in wheat flour,[3] and the depletion of glutathione during dough mixing and fermentation may result from enzymatic oxidation of GSH. Each of the mixing steps in a multistage sourdough fermentation aerates the dough[22] and promotes oxidation of glutathione to GSSG.[3] Glutathione as well as GSSG undergoes sulfhydryl/disulfide bond changes with gluten proteins but has opposite effects on gluten strength.[3] Glutathione depolymerizes gluten protein by binding to those cysteine residues that mediate intermolecular disulfide bonds in the gluten macropolymer.[3,31] GSH is the most abundant low molecular weight thiols in flour; others include Glu-Cys, cysteine, and Cys-Gly.[3]

Sourdough fermentation with *L. sanfranciscensis* increased thiol levels in wheat sourdoughs,[10,19] but the impact of *L. sanfranciscensis* on the glutathione concentration in sourdough has not been reported. Moreover, bacterial glutathione reduction has previously not been compared to glutathione release by yeasts. *L. sanfranciscensis* employs NADH oxidase as well as glutathione reductase, thioredoxin reductases, and a cyst(e)ine transport protein to resist oxidative stress;[32] these enzymatic activities all affect thiol levels in sourdoughs. By comparison of *L. sanfranciscensis* wild type and GshR deficient mutant strains, this study demonstrates that glutathione reductase directly influences glutathione and γ-glutamyl cysteine concentrations in sourdough (Figures 2 and 3). Differences between GshR positive and negative strains of *L. sanfranciscensis*, however, were smaller than differences between sourdoughs fermented with *L. sanfranciscensis* and chemically acidified or yeast fermented controls, demonstrating that other metabolic activities that consume oxygen or generate low molecular weight thiol compounds are equally important. The genome of *L. sanfranciscensis* does not code for a γ-glutamyl-cysteine synthetase;[25] therefore, accumulation of γ-glutamyl cysteine reflects enzymatic degradation of glutathione rather than microbial biosynthesis or protein degradation. Wheat glutathione dehydrogenase converts glutathione to the oxidized dimer but does not recognize γ-glutamyl cysteine as substrate.[3] Reduced γ-glutamyl-cysteine is thus not subject to enzymatic reoxidation during dough mixing. Accordingly, differences between γ-glutamyl cysteine concentrations in sourdough fermented with *L. sanfranciscensis* wild type and GshR mutant strains were more significant than differences in glutathione concentrations.

Influence of Sourdough on Bread Taste. Glutathione is a kokumi-active compound, which enhances continuity and mouthfulness when added to a solution containing glutamate and inosine-5′-monophosphate.[33,34] Glutathione impacts the taste of seafood, beef extract, and chicken broth,[33,35] but its role in bread taste has not been documented. The concentration of glutathione in *L. sanfranciscensis* sourdough bread, 56 μmol kg^{-1}, and the difference in glutathione concentration relative to control bread, less than 30 (Figure 3), were much lower than the taste threshold of 0.3 mmol L^{-1}.[34] Glutamate accumulation by lactobacilli sourdough influenced the taste of bread only if the concentration difference in bread exceeded the taste threshold of 1 mmol kg^{-1}.[12] Accordingly, fermentation with glutathione accumulating *L. sanfranciscensis* did not influence the intensity of the salty taste when compared to regular bread. Remarkably, *L. sakei* did not accumulate glutathione, but the intensity of the salty taste of sourdough bread produced with *L. sakei* was significantly higher when compared to regular bread. Comparable effects of acidity on the perceived salty taste of bread were previously reported with chemically acidified bread as well as sourdough bread.[12,36]

Effect of Glutathione on Bread Volume and Texture. The impact of exogenous addition of reduced glutathione on dough rheology and bread volume is well documented;[3] however, the influence of glutathione reductase activity of

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sourdough microbiota has not been investigated.[11] This study compared the impact of *L. sanfranciscensis* wild type and isogenic GshR deficient strains to provide direct evidence for the effect of glutathione reductase on bread volume and texture. Yeast was added to obtain a reproducible yeast inoculum. Although the metabolic activity of yeasts may influence the accumulation of glutathione, comparison of the *L. sanfranciscensis* wild type and its isogenic *gshR* deficient mutant allows attribution of effects on bacterial glutathione metabolism. Bread produced with *L. sanfranciscensis* had a lower specific volume than bread produced with the isogenic mutant; sensory evaluation of bread texture confirmed that *L. sanfranciscensis* sourdough bread is significantly harder than the *L. sanfranciscensis* Δ*gshR* fermented bread. Glutathione promotes SH/SS interchange reactions leading to a weaker dough, reduced mixing time and improved dough machinability,[37] however, the effect of glutathione is concentration dependent.[38] Remarkably, fermentation with *L. sanfranciscensis* Δ*gshR* significantly increased bread volume when compared to yeast leavened bread or in comparison to the wild type strain. Glutathione concentrations in bread dough fermented with the *L. sanfranciscensis* wild type and GshR mutant strains match the levels that were previously reported to decrease and increase, respectively, the volume of bread.[38] The beneficial effect of *L. sanfranciscensis* Δ*gshR* on bread volume also confirms the hypothesis that exopolysaccharide production as well as the solubilization of flour polysaccharides (partially) compensate the reduced gluten strength in wheat sourdoughs.[11,39,40] The present study employed bleached flour with a relatively high gluten content. This emphasizes the role of glutathione in reducing bread volume; the relevance of flour polysaccharides and microbial exopolysaccharides for dough hydration and bread volume is more pronounced with unbleached flour or low-gluten flours.[39]

In conclusion, this study developed a fermentation protocol to mimic traditional type I sourdough fermentations that are used as a sole leavening agent. The use of *L. sanfranciscensis* wild type strain and its isogenic and GshR deficient mutant demonstrated that glutathione reductase activity in sourdough lactobacilli contributes to the accumulation of glutathione in bread and may negatively impact bread volume. The comparison of the effect of *L. sanfranciscensis* wild type and mutant strains also demonstrated that other metabolic activities of *L. sanfranciscensis* increase the loaf volume when compared to bread produced with a straight dough process. Artisanal sourdough bread is valued by customers for its superior taste and aroma; however, this study indicates that further fine-tuning of ingredients, fermentation microbiota, and fermentation conditions may improve the loaf volume of sourdough bread produced with *L. sanfranciscensis*.

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