Characterization of γ-Glutamyl Cysteine Ligases from *Limosilactobacillus reuteri* Producing Kokumi Active γ-Glutamyl Dipeptides

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Abstract

γ-Glutamyl cysteine ligases (Gcl) catalyse the first step of glutathione synthesis in prokaryotes and many eukaryotes. This study aimed to determine the biochemical properties of three different Gcls from strains of *Limosilactobacillus reuteri* that accumulate γ-glutamyl dipeptides. Gcl1, Gcl2 and Gcl3 were heterologously expressed in *Escherichia coli* and purified by affinity chromatography. Gcl1, Gcl2 and Gcl2 exhibited biochemical with respect to the requirement for metal ions, the optimum pH and temperature of activity, and the kinetic constants for the substrates cysteine and glutamate. The substrate specificities of the three Gcls to 14 amino acids were assessed by liquid chromatography – mass spectrometry. All three Gcls converted ala, met, glu, and gln into the corresponding γ-glutamyl dipeptides. None of the three were active with val, asp and his. Gcl1 and Gcl3 but not Gcl2 formed γ-glu-leu, γ-glu-ile and γ-glu-phe; Gcl3 exhibited stronger activity with gly, pro and asp when compared to Gcl2. Phylogenetic analysis of Gcl and the Gcl-domain of GshAB in lactobacilli demonstrated that most of Gcls were present in heterofermentative lactobacilli while GshAB was identified predominantly in homofermentative lactobacilli. This distribution suggests a different ecological role of the enzyme in homofermentative and heterofermentative lactobacilli. In conclusion, three Gcls exhibited similar biochemical properties but differed with respect to their substrate specificity and thus the synthesis of kokumi-active γ-glutamyl dipeptides.

Keywords: γ-glutamyl cysteine ligases, *Limosilactobacillus reuteri*, characterization, γ-glutamyl dipeptides, kokumi activity

Key Points

Strains of *Limosilactobacillus reuteri* encode for up to 3 glutamyl-cysteine ligases

Gcl1, Gcl2 and Gcl3 of *Lm. reuteri* differ in their substrate specificity

Gcl1 and Gcl3 produce kokumi-active dipeptides.

Introduction

γ-Glutamyl peptides are potent bioactives which interact with vertebrate Calcium-sensing proteins (Yang et al. 2019). When present in micromolar concentrations, γ-glutamyl peptides modulate signaling cascades that relate to taste perception and inflammation (Ohsu et al. 2010; Zhang et al. 2015). *In vivo* anti-inflammatory activity was demonstrated for dietary γ-Glu-Val (Zhang et al. 2015; Zhang et al. 2016). γ-Glutamyl-dipeptides were also act as taste enhancers with kokumi taste activity (Ueda et al. 1994; Toelstede and Hofmann 2009a) which is described as "mouthfulness", "continuity" and "thickness" (Beksan et al. 2003; Sforza et al. 2006; Toelstede and Hofmann 2009a; Toelstede et al. 2009). Several γ-glutamyl dipeptides are known to be kokumi-active including γ-Glu-Glu, γ-Glu-Gln, γ-Glu-Gly, γ-Glu-Ala, γ-Glu-Val, γ-Glu-Met, γ-Glu-Leu, γ-Glu-Phe, and γ-Glu-His. The threshold for kokumi activity was reported to range from 5 µmol / kg to 2.5 mmol / kg, however, a standardized protocol for determination of the threshold for kokumi-activity through sensory analyses is not available. Moreover, the kokumi threshold depends on the food matrix in which kokumi-activity is determined (Stark and Hofmann 2005; Glabasnia and Hofmann 2006; Toelstede et al. 2009; Yang et al. 2019). Kokumi-active γ-glutamyl peptides occur in wheat (Sarwin et al. 1992), edible beans (Liao et al. 2013), garlic (Nakamoto et al. 2018) and onions (Ueda et al. 1994). In addition to their presence in plants, microbial activity generates γ-glutamyl dipeptides in food fermentation including ripened cheese (Toelstede and Hofmann 2009a), soy sauce (Kuroda and Miyamura 2015) and sourdough for bread-making (Zhao and Gänzle 2016). Because kokumi-active compounds substantially contribute to the taste of some foods, they are an attractive tool for food product development and may allow reduction of the salt and sugar content of foods without compromising consumer acceptance (Zhao and Gänzle 2016; Yang et al. 2019).

Microbial enzymes involved in the synthesis of γ-glutamyl dipeptides include γ-glutamyl transferase/transpeptidase (GGT, EC 2.3.2.2, Suzuki et al. 2007), glutaminase (EC 3.5.1.2, Nandakumar et al. 2003) and γ-glutamyl-cysteine ligase (EC 6.3.2.2, Roudot-Algaron et al. 1994). The transpeptidase activity of GGT generates γ-glutamyl dipeptides *in vitro* and food fermentations (Toelstede and Hofmann 2009b; Hillmann et al. 2016). At pH-values higher than 7.5, fungal and bacterial glutaminases catalyze the formation of γ-glutamyl dipeptides from glutamate and amino acids *in vitro* (Tomita et al. 1989; Tachiki et al. 1996; Yang et al. 2017) but glutaminases of lactobacilli do not contribute to formation of kokumi-active compounds in food fermentations (Li et al. 2020). Evidence for the contribution of Gcl is provided by the strain-specific accumulation of γ-glutamyl dipeptides in sourdough fermentation with *Limosilactobacillus reuteri* (Zhao and Gänzle 2016). The role of two Gcls in the biosynthesis of several γ-glutamyl dipeptides has been confirmed with Gcl-deficient mutant strains of *Lm. reuteri* LTH5448, indicating that *gcl1* and *gcl2* were responsible for γ-Glu-Ile and γ-Glu-Cys synthesis, respectively (Yan et al. 2018).

γ-Glu-Cys ligases (Gcls) are also the rate-limiting enzyme in the synthesis of glutathione (GSH). Gcls synthesize γ-glu-cys in an ATP-dependent reaction by ligating the γ-carboxyl group of glutamate to cysteine (Figure 1). Gcls have been characterized from several bacteria (Kelly et al. 2002; Vergauwen et al. 2006; Kino et al. 2007) and yeast (Kong et al. 2018). Gcl domains of GshAB from *Streptococcus agalactiae* and *Escherichia coli* catalyze the biosynthesis of several γ-glutamyl dipeptides (Figure 1). Heterofermentative lactobacilli frequently harbor *gcl* genes and genomes of strains in the genera *Lentilactobacillus* and *Limosilactobacillus* frequently encode for two or three *gcl* genes. In these organisms, the function of Gcls is not related to glutathione synthesis because genes coding for GSH synthetase (GS, EC 6.3.2.3) are generally absent (Pophaly

et al. 2012; Zheng et al. 2015; Yan et al. 2018). However, few studies characterized the activity of Gcl enzymes of food-fermenting lactobacilli to elucidate their contribution to synthesis of γ-glutamyl dipeptides. It was therefore the aim of this study to characterize three Gcls from *Lm. reuteri* and assess their contribution in the synthesis of kokumi-active γ-glutamyl dipeptides.

Materials and Methods

Bacteria Strains and Growth Conditions.

Lm. reuteri subsp. *murium* LTH5448, which encodes for Gcl1 and Gcl2, and *Lm. reuteri* subsp. *rodentium* LTH2584, which encodes for Gcl1, Gcl2, and Gcl3, were grown at 37 °C in modified deMan-Rogosa-Sharpe (mMRS) medium with the following ingredients per liter: 5 g beef extract, 5 g yeast extract, 10 g peptone, 10 g malt extract, 10 g maltose, 5 g fructose, 5 g glucose, 2.6 g KH_2PO_4, 4 g K_2HPO_4, 3 g NH_4CL, 0.5 g cysteine HCl, 1 g Tween 80, 0.1 mg $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot H_2O$. *E.coli* DH5α and *E. coli* BL21 star (DE3), which were used as a host for the construction of plasmids pET-28a(+) with the insert gene and the over-expression of recombinant proteins, respectively, were grown in Luria-Bertani (LB) medium (BD, Mississauga, CA, U.S.A.) at 37 °C with shaking. Kanamycin (50 mg/L) was added into LB medium for *E. coli* growth carrying plasmids pET-28a(+).

Chemicals and Reagents.

2,3-Naphthalenedicarboxaldehyde (NDA), ATP, 5-sulfosalicylic acid (SSA), Me_2SO, L-amino acids and other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). γ-Glutamyl dipeptides (γ-glu-ala, γ-glu-leu, γ-glu-ile, γ-glu-phe, γ-glu-met, γ-glu-pro, γ-glu-gly, γ-glu-ser, γ-glu-glu, γ-glu-gln, and γ-glu-asp) were obtained from United Biosystems (Herndon, VA, U.S.A.).

DNA Manipulations.

The Blood & Tissue Kit (Qiagen, Hilden, Germany) and GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Scientific, Mississauga, CA, U.S.A.) were used for the isolation of genomic DNA and the extraction of plasmid DNA, respectively. Primers were obtained from Integrated DNA Technologies (San Diego, CA, U.S.A.). Phusion High-Fidelity DNA polymerases, T4 DNA ligase and restriction enzymes were obtained from Thermo Scientific. PCR fragments were purified with GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Scientific). Sanger sequencing of PCR products was performed by the Molecular Biology Service Unit (MBSU) of the Department of Biological Sciences at the University of Alberta.

Cloning and Heterologous Expression of Gcl from *Lm. reuteri*.

The *gcl* genes (Gcl1, WP_035156810.1; Gcl2, WP_085680095.1; Gcl3, KEK14969) from *Lm. reuteri* LTH5448 and LTH2584 were amplified with primers shown in Table 1. The *gcl* PCR fragments and expression vector pET-28a(+) were purified, digested with the same restriction endonucleases (Table 1), and ligated into plasmids pET-28a(+)/*gcl* using T4 DNA ligase. Recombinant plasmids were; transformed into *E. coli* DH5α and sequences of the inserted *gcls* were verified by DNA sequencing. The resulting plasmids pET-28a(+) with the *gcl* genes were extracted from *E. coli* DH5α and introduced into the expression host *E. coli* BL21 star (DE3). *E. coli* BL21 (DE3) strains with pET-28a (+) carrying *gcl1*, *gcl2* or *gcl3* were incubated in LB broth at 37 °C with 180 rpm agitation. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.2 mM) was added to induce the over-expression of recombinant proteins when the optical density (OD) of the culture at 600nm was between 0.4 and 0.6, followed by further incubation for 18 h at 25 °C. Cells were harvested by centrifugation at 4 °C and stored at -80 °C until use.

Purification of Gcl1, Gcl2, and Gcl3.

Gcl1 and Gcl2 were over-expressed as soluble proteins while the over-expressed Gcl3 was present in inclusion bodies (data not shown). The purification of soluble Gcl1 or Gcl2 was performed by HisPur Ni-NTA spin column (Thermo Scientific) according to the instructions of the supplier. The proteins were finally eluted into 50 mM Tris-HCl buffer at pH 8.0 and stored in 15% glycerol at -20 °C until use. Inclusion bodies containing Gcl3 inclusion bodies were solubilized and refolded by using the protein refolding kit (Novagen) according to the manufacturer's instruction. Refolded Gcl3 was then concentrated with a 10 KDa centrifugal filter unit (Merck Millipore Ltd., Carrigtwohill, Ireland) and stored in 15% glycerol at -20 °C until use. The enzymatic activity of three Gcls remained stable over 6 months of storage (data not shown).

Biochemical characterization of Gcls

Biochemical characteristics of the three Gcl enzymes were assayed with a microtitre-plate based fluorescence method to quantify γ-glu-cys (White et al. 2003). After precipitating proteins with SSA, the microtitre-plates were kept on ice for 20 min and the precipitate was removed by centrifugation. Aliquots of 20 µl of the reaction supernatant were transferred to a 96-well black plate (Corning Incorporated, Kennebunk, U.S.A) and mixed with 180 µl of NDA derivatization solution(White et al. 2003). The plate was covered and incubated in dark for 30 min at ambient temperature. The fluorescence intensity of NDA-γ-glu-cys or GSH was determined with an excitation wavelength of 472 nm and an emission wavelength of 528 nm with a fluorescence plate reader (Varioskan Flash, Thermo Electron Corporation, CA, USA). A standard curve for quantification of γ-glu-cys was generated with glutathione (GSH).

To assay the inhibition of Gcl by L-buthionine-sulfoximine (L-BSO), 5µl of L-BSO solution was added as a final concentration (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 1.0 mM, or 2.0 mM) into the mixture of 75 µl of reaction cocktail (50 mM Trizma Base, 40 mM ATP, 20 mM

Glu, 40 mM MgCl2, and 2 mM EDTA, pH 8.0) with 25 µl of enzyme. The addition of 5 µl of H_2O without L-BSO was used as reference. The reaction mixtures with L-BSO or water were pre-incubated for 5 min at 37 °C, followed by pipetting 50 µl of 5 mM of cysteine solution (pH 8.0) to initiate the enzymatic reaction. The reactions were carried out for 45 min at 37 °C and stopped by 50 µl of 500 mM SSA. In a reaction control, the cysteine solution was not pipetted into the mixture until the addition of SSA.

To determine the effect of metal ions on the Gcl activity, 5µl of metal ion solutions (400 mM of Mg^{2+} or 100 mM of all other metal ions) were pipetted into 100 µl of the reaction mixture (pH 8.0) without metal ions. Addition of 5 µl of water served as control. After a pre-incubation for 5 min at 37 °C, 50 µl of 5 mM of cysteine solution (pH 8.0) was used to initiate the reaction. Following an additional incubation for 45 min at 37 °C, 50 µl of 500 mM SSA was added to terminate the reaction. For each metal ion, the corresponding controls were carried out in parallel.

To determine the optimal pH of enzymatic reaction by Gcl, reaction cocktails (40 mM ATP, 20 mM Glu, 40 mM MgCl2, and 2 mM EDTA) were prepared with pH values ranging from 4.0 to 10.5. The following buffers were used: 50 mM of citrate buffer (pH 4.6 0 6.0), 50 mM of Tris-HCl buffer (pH 7.0 – 9.0) and 50 mM of carbonate-bicarbonate buffer (pH 9.5−10.5). Cysteine was dissolved in the buffer corresponding to the pH of reaction cocktail to a final concentration of 5 mM cysteine. The reaction mixtures containing 75 µl of reaction cocktail and 25 µl of Gcl enzyme were pre-warmed for 5 min at 37 °C, then mixed with 50 µl of 5 mM of cysteine solutions to start the reaction, followed by incubation for 45 min at 37 °C. The reaction was stopped by the addition of 50 µl of 500 mM SSA. Reaction controls where cysteine was added only after addition of SSA were conducted for each pH.

To assay the effect of temperature on the activity of Gcl, the enzymatic reaction was carried out at different temperature ranging from 10 to 70 °C. 25 µl of Gcl enzyme was pipetted into the reaction cocktail (50 mM Trizma Base, 40 mM ATP, 20 mM Glu, 40 mM $MgCl_2$, and 2 mM EDTA, pH 9.0), then pre-incubated for 5 min at desirable temperature. Following that, 50 µl of 5 mM of cysteine solution (pH 9.0) was added to start the enzymatic reaction, which was terminated with 50 µl of 500 mM SSA. Reaction controls were performed at each temperature. Results are reported as means ± standard deviation of three biological replicates.

Kinetic Characteristics of Gcl with Substrate Cysteine and Glutamate

The kinetic constants K_m and V_{max} of three Gcl enzymes for the two substrates cysteine and glutamic acid were measured in this study. To assay the kinetic characteristic of Gcl for substrate cysteine, a series of cysteine solutions (pH 9.0) were added to obtain a final concentration of 0.03, 0.10, 0.17, 0.33, 0.50, 0.67, 1.33, 2.00 or 3.33 mM. The concentration of glutamate was 10 mM. Similarly, kinetic constants of Gcl enzymes for glutamate were studies by addition of 50 µl of glutamate solutions (pH 9.0) to a final concentration of 0.3, 1.0, 2.0, 3.0, 5.0, 10.0, 20.0 or 30.0 mM into the mixtures of 75 µl of reaction cocktail (50 mM Trizma base, 40 mM ATP, 5 mM Cys, 40 mM MgCl2, and 2 mM EDTA, pH 9.0) with 25 µl of enzyme. All reactions were performed at 43 °C, pH 9.0 with 45 min incubation, then terminated by using 50 µl of 500 mM SSA. The concentration of γ-glu-cys was assayed using fluorescence-based microtiter plate assay. The experimental data of the enzymatic activity was fitted to the Michaelis-Menten equation:

$$V = \frac{V_{max}[S]}{K_m + [S]},$$

where V is the reaction velocity, V_{max} is the maximum reaction velocity, [S] is the substrate concentration and K_m is the Michaelis-Menten constant. The parameters V_{max} and K_m were

estimated with the non-linear curve fit tool implemented in SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA, U.S.A). Results are reported as means ± standard deviation of three biological replicates.

Qualitative assessment of the substrate specificity of Gcls by LC-MS/MS analysis

To assess the substrate specificity of Gcls, 50 µl of amino acid solution (pH 9.0) containing 20 mM of ala, gly, ser, leu, ile, val, phe, met, cys, pro, glu, gln, asp or his was added into the reaction mixtures of 75 µl of reaction cocktail (50 mM Trizma base, 40 mM ATP, 60 mM Glu, 40 mM $MgCl_2$, and 2 mM EDTA, pH 9.0) with 25 µl of enzyme. All reactions were carried out for 2 h at 43 °C where the highest yield of dipeptides was obtained with glu and cys as substrates (data not shown).

γ-Glutamyl dipeptides were separated using an Agilent 1100 series HPLC unit equipped with a Luna Omega polar C18 column (1.6 µm, 50 × 2.1 mm, Phenomenex, Torrance, CA, U.S.A.) and detected using a Micromass Quattro micro API tandem quadrupole LC-MS/MS system (Waters Corporation, Milford, Massachusetts, U.S.A) with multiple reaction monitoring (MRM) mode. Mobile phase A and B consisted of 0.1 % formic acid in Milli-Q water and 0.1 % formic acid in acetonitrile, respectively. Samples were eluted at a flow of 0.3 ml/min as the following gradients: 0−6.50 min, 100−88% A; 6.50−6.51 min, 88−25% A; 6.51−8.00 min, 25% A; and followed by a column re-equilibration with 100% A for 8 min at a flow of 0.35 ml/min. Data acquisition was interfaced to the Masslynx v4.1 software (Waters Corporation, Milford, Massachusetts, U.S.A).

Phylogenetic Analysis and Gene Clustering.

Three putative genes coding for Gcl were identified in *Lm. reuteri* (Yan et al. 2018). Two of these sequences, *gcl1* and *gcl2*, are present in the genome of *Lm. reuteri* LTH5448 while *Lm. reuteri*

LTH2584 additionally harbors *gcl3*. The protein sequences of glutamyl cysteine ligases (Gcl) and GSH synthetase (GshAB) in all lactobacilli were retrieved from NCBI database using protein sequences of Gcl1, Gcl2 and Gcl3 as a query sequence. One sequence per protein (Gcl or GshAB) from the same species was selected with a cutoff of 25% amino acid identity; if similar sequences were present in the same species, the sequence with a lower amino acid identity was removed. A total of 221 protein sequences of Gcl and GshAB were selected, then aligned by Muscle in MEGAX. The phylogenetic analysis with the aligned sequences was carried out by IQ-Tree software (iqtree.cibiv.univie.ac.at) using the maximum likelihood method. LG+F+G4 was found as a best-fit model. The phylogenetic tree was displayed in iTOL.

Statistical Analysis

Data analysis for the relative activity in the inhibition experiment by L-BSO was performed by one-way analysis of variance (ANOVA) using IBM SPSS statistics 23; values were considered significantly different at a 5% error level ($p < 0.05$). P value of ≤ 0.05 with Tukey adjustment for multiple comparisons was considered statistically significant.

Results

Expression and Purification of Glutamyl Cysteine Ligases from *Lm. reuteri*

To determine whether different glutamyl cysteine ligases that are present in the same genome of strains of *Lm. reuteri* differ with respect to their biochemical properties, Gcl1, Gcl2 and Gcl3 were purified after heterologous expression in *E. coli*. SDS-PAGE analysis of purified Gcl1 and Gcl2 demonstrated a single band which was absent in the crude cellular extracts of *E. coli* with the empty plasmid pET28a (+) (Figure 2). SDS-PAGE analysis of Gcl3, which was purified from inclusion bodies, indicated the presence of the refolded Gcl3 as major band together with few other

faint bands (Figure 2). The molecular weights of Gcl1, Gcl2 and Gcl3 as estimated by SDS-PAGE was approximately 5 kDa larger than the predicted molecular weight of 51.10, 59.29 and 49.34 kDa (www.bioinformatics.org/sms/prot_mw.html), respectively.

The pairwise amino acid identity of Gcl domains from Gcl1, Gcl2 and Gcl3 are shown in Table 2. The 337 amino acid Gcl domain of Gcl2 was 31.1% identical to the Gcl domain of Gcl1 (315 amino acids) and 28.0% identical to the Gcl domain of Gcl3 (314 amino acids). The Gcl domains of Gcl1 and Gcl3 were more than 40% identical. *S. agalacticae* harbors *gshAB* encoding two-domain GSH synthetase (Janowiak and Griffith 2005) and the Gcl domain of GshAB (320 amino acids) was 33−35% identical to the Gcl domains of *Lm. reuteri* enzymes (Table 2).

Inhibition of Gcl by L-buthionine-sulfoximine (L-BSO)

L-BSO inhibits Gcl enzymes by binding to the active site of Gcl, which disturbs binding of cysteine (Kelly et al. 2002; Hibi et al. 2004; Janowiak and Griffith 2005). To measure the inhibition of Gcl1, Gcl2 and Gcl3 by L-BSO, the reactions for these three enzymes were performed in presence of different concentrations of L-BSO. The results indicated that 0.3 mM L-BSO reduced activity of the three Gcls from *Lm. reuteri* by 50% (Figure 3). The inhibition of Gcl1 and Gcl2 was similar but activity of Gcl3 was further reduced by 75% if the concentration of L-BSO was increased to 0.6 mM. These results suggest that inhibition of Gcls from *Lm. reuteri* by L-BSO is less pronounced when compared to Gcl from *E. coli* or mammalian Gcls (Griffith 1982; Kelly et al. 2002).

The Effect of Metal Ions on the Gcl Activity

In the reaction catalyzed by the ATP-dependent glutamyl cysteine ligase, divalent metal ions play a key role in ATP binding, phosphoryl transfer, the stabilization of the structure of

γ-glutamylphosphate intermediate in transition state and the elimination of ADP and phosphate (Abbott et al. 2001; Hibi et al. 2004). Particularly Mg^{2+} and Mn^{2+} are key co-factors for the activity of Gcl enzymes. To assay the role of metal ions in the activity of *Lm. reuteri* Gcls, Mg^{2+}, Mn^{2+} and 9 other metal ions were used in enzymatic reactions, respectively (Figure 4). Gcls from *Lm. reuteri* Gcls were inactive unless in the absence of Mg^{2+} or Mn^{2+}. Other metal ions, including K^{+}, Ca^{2+}, Zn^{2+}, Ba^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+}, Fe^{2+} and Fe^{3+}, did not support activity of Gcls of *Lm. reuteri*. The activity of Gcls in presence of Mg^{2+} was substantially higher than the activity in presence of Mn^{2+}, however, the concentration of Mn^{2+} was 4-fold lower than that of Mg^{2+} because higher concentrations of Mn^{2+} precipitated all three Gcl proteins (data not shown).

Determination of the optimal pH and Temperature of the Enzymatic Reaction by Gcls

Gcl1, Gcl2 and Gcl3 were characterized with regards to their activity at pH 4.0 – 10.5 and in the temperature range of 10 – 70 ° (Figure 5). All three *Lm. reuteri* Gcl enzymes were optimally active at pH 9.0; the minimal pH was 6.0 for all three enzymes. Gcl3 was less active than Gcl1 and Gcl2 at pH values higher than pH 9.0. The activities of Gcl1 and Gcl2 were also significantly reduced at a pH of 10.5. Gcl1, Gcl2 and Gcl3 showed a similar response to the reaction temperature. The optimal temperature of all three Gcl enzymes was 50 °C, and 90% relative activity was achieved when performing the reaction at 43−60 °C. Gcl1, Gcl2 and Gcl3 remained active at 10 °C but were completely inactivated at 70 °C.

Kinetic Characteristics of Gcls with Substrate Cysteine and Glutamate

Cysteine and glutamate are the substrates for synthesis of γ-glu-cys by Gcls. For determination of the K_m and V_{max} values of Gcls for cysteine or glutamate, other substrates and co-factors for Gcl activity were added to saturation. The concentration of γ-glu-cys synthesized by Gcls linearly

increased for the first 60 min of the reaction (data not shown). A 45 min incubation time was thus utilized to determine the kinetic constants for Glu and Cys. The experimental data was fitted to the Michaelis-Menten kinetics and difference between experimental and predicted values was smaller than the experimental error for most data points (Figure 6). The K_m values for Gcl1, Gcl2 and Gcl3 with cysteine substrate were 0.11, 0.10 and 0.18 mM, respectively, and the K_m–values for the substrate glutamate were 1.54, 1.46 and 1.56 mM, respectively. All three enzymes thus exhibited a 10-fold higher affinity for cysteine than for glutamate. Gcl3 exhibited higher V_{max} values with cysteine and glutamate than Gcl1 and Gcl2.

Substrate Specificity of *Lm. reuteri* Gcls

To evaluate the activity of Gcls with amino acids other than cysteine as second substrate, the reaction was performed with glutamate and one each of 14 amino acids (Table 3). The amino acids were chosen to include the substrates for the 10 γ-glutamyl dipeptides with demonstrated kokumi activity (Zhao et al. 2016), and to additionally include polar and charged amino acids. Of the amino acids tested, alanine, methionine, cysteine, glutamate and glutamine were good acceptors for all three Gcls. None of the three enzymes was active with aspartate, valine and histidine. Gcl3 exhibited a broader substrate specificity compared to Gcl1 and Gcl2 and produced γ-glutamyl dipeptides with glycine, serine and proline, which were not observed in reactions with Gcl1 and Gcl2. Gcl2 differed from Gcl1 and Gcl3 as the hydrophobic amino acid leucine, isoleucine and phenylalanine were not used as substrates to form the corresponding γ-glutamyl dipeptides.

Phylogenetic Analysis of Glutamyl Cysteine Ligases and Two-Domain GSH Synthetases in Lactobacilli

To clarify the phylogenetic relationships of three putative *Lm. reuteri* Gcls and all other Gcls and GshABs in lactobacilli (Zheng et al. 2020), Gcl and GshAB sequences of lactobacilli were aligned

to construct a phylogenetic tree (Figure 7). The tree displays four clusters that comprise sequences of Gcl1 and Gcl3, a family of sequences that consisted of GshAB and related Gcls, Gcl2 sequences, and additional Gcl sequences that were mainly present in the genus *Levilactobacillus* and *Lentilactobacillus*. Most of Gcl sequences were present in heterofermentative lactobacilli while most GshAB sequences were present in homofermentative lactobacilli. GshAB sequences clustered separately from Gcl sequences. Gcl2 sequences were mainly identified in strains of the genus *Limosilactobacillus* and clustered separately from Gcl1 and Gcl3. The four Gcl3 sequences from strains in the genus *Limosilactobacillus* clustered separately from Gcl1 sequences in the genera *Limosilactobacillus, Ligilactobacillus* and *Lentilactobacillus* (Figure 7) but the phylogenetic tree supports the close relationship of Gcl1 and Gcl3 that is also suggested by the pairwise amino acid identity (Table 2).

Discussion

γ-Glutamyl dipeptides improve food quality as kokumi-active compounds (Toelstede et al. 2009; Zhao and Gänzle 2016) and additionally relieve gut inflammation (Zhang et al. 2015; Zhang et al. 2016). This study charracterized three Gcl enzymes from *Lm. reuteri* that synthezise γ-glutamyl dipeptides.

All three Gcls of *Lm. reuteri* were inhibited by L-BSO, as was previously reported for Gcl enzymes (Kelly et al. 2002; Janowiak and Griffith 2005). The inhibition efficiency of L-BSO to three *Lm. reuteri* Gcls was similar to Gcl domain of GshAB from *S. agalacticae* (Janowiak and Griffith 2005) but L-BSO was less inhibitory to *Lm. reuteri* Gcls and *S. agalacticae* Gcl when compared to Gcls from *E. coli* or eukaryotes (Kelly et al. 2002). The reduced inhibition by L-BSO has been related to a lower binding affinity of L-BSO to Gcls (Janowiak and Griffith 2005). Overall, the results for

three the Gcls of *Lm. reuteri* and *S. agalacticae* indicate that L-BSO is less inhibitory for Gcls of *Lactobacillales* when compared to Gcl enzymes from other bacteria.

Gcl as an ATP-dependent ligase requires a divalent metal ion for catalysis. Divalent metal ions not only serve as an essential co-factor to activate Gcl but also change its substrate specificity (Orlowski and Meister 1971; Kelly et al. 2002). Addition of Mg^{2+} or Mn^{2+} was an absolute requirement for the activity of Gcls in *Lm. reuteri*, in keeping with the effect of these ions on activity of other Gcls (Abbott et al. 2001; Janowiak and Griffith 2005). Gcls of *Lm. reuteri*, however, were not active in presence of Co^{2+}, Cd^{2+} and Fe^{2+} while these metal ions activated Gcl of *E. coli* (Kelly et al. 2002).

Although the activity of Gcl has typically been assayed at pH 8.0 − 8.5 and 37 °C (Abbott et al. 2001; Kelly et al. 2002; Kino et al. 2007), the optimal pH and temperature of the three *Lm. reuteri* Gcls were 9.0 and 50 °C. The synthesis of γ-glutamyl dipeptides by Gcl involves a transpeptidation reaction where the donor γ-glutamyl moiety is transferred to an acceptor nucleophile, i.e. the second amino acid. With regards to the optimum pH of the transpeptidation activity, Gcls are similar to GGT and glutaminase that catalyze γ-glutamyl transfer at alkaline pH in the range of 7.5 − 11 (Tachiki et al. 1998; Nandakumar et al. 2003; Suzuki et al. 2007; Morelli et al. 2014).

All three *Lm. reuteri* Gcls exhibited a higher affinity for cysteine than glutamate, consistent with properties of Gcls in humans (Thulin and Linse 1998), *E. coli* (Kelly et al. 2002), *S. agalacticae* (Janowiak and Griffith 2005) and *Pseudoalteromonas haloplanktis* (Albino et al. 2014). The high affinity of Gcls for cysteine may be related to intercellular levels of cysteine. *In vivo* concentrations of cysteine are maintained at relatively low levels (Lee et al. 2004; Stipanuk 2004). The K_m values of *Lm. reuteri* Gcls for glutamate were similar to each other and to those of human or *E. coli* but 10-fold lower than those of *S. agalacticae* (Janowiak and Griffith 2005).

The substrate specificity of Gcls or GshAB was previously determined with enzymes from *E. coli* or *S. agalacticae*. These previously characterized Gcls or GshAB exhibited different substrate specificities (Kino et al. 2007). The present study provides an unprecedented characterization of Gcl activity in food fermenting bacteria. Although three *Lm. reuteri* Gcls showed overlapping substrate specificities, Gcl3 had the broadest substrate spectrum among three enzymes. Met and cys were good acceptors for Gcl1, Gcl2 and Gcl3. Met was also a good γ-glutamyl acceptor for *S. agalacticae* GshAB (Kino et al. 2007) and GGT from *Penicillium roqueforti* (Toelstede and Hofmann 2009b) but not for Glc from *E. coli* (Kino et al. 2007). Similar to Gcl from *E. coli* and GGT, Gcl from *Lm. reuteri* was not active with histidine (Toelstede and Hofmann 2009b). In contrast, γ-glu-his was synthesized by *S. agalacticae* GshAB (Kino et al. 2007). Gcl1 and Gcl3 were active with the hydrophobic amino acid leu, ile and phe but Gcl2 was not. Differences of the substrate-specificity between Gcl2 and Gcl1 or Gcl3 relate to their phylogenetic relationship and low amino acid identities, with Gcl2 being most distant from Gcl1 and Gcl3. Gcl3 and Gcl1 are 43% identical but they differed in their substrate specificity, indicating that the specificity of uncharacterized Gcls in *Levilactobacillus* and *Lentilactobacillus* species may also differ.

The distribution of *gcl* or *gshAB* in lactobacilli indicates that Gcl has a different physiological role in heterofermentative and homofermentative lactobacilli. Homofermentative organisms harbor predominantly GshAB, indicating that Gcl acts as part of the GSH biosynthetic pathway to protect against oxidative stress (Fu et al. 2006; Li et al. 2011). In heterofermentative lactobacilli, GSH also protects against environmental stress including oxidative and cold stress (Jänsch et al. 2007; Zhang et al. 2010) but most heterofermentative lactobacilli lack the complete pathway for glutathione synthesis (Pophaly et al. 2012). The absence of GSH synthetase activity of GshAB in heterofermentative lactobacilli is compensated by transport and reduction of GSH or GSSG

(Jänsch et al. 2007; Pophaly et al. 2012) or by uptake of cysteine and / or methionine (Lo et al. 2009; Stetina et al. 2014). The activity of Gcls in *Lm reuteri* appeared to be unrelated to the resistance to oxidative stress (Yan et al. 2018) and the role of multiple Gcls in the ecology of heterofermentative lactobacilli remains to be investigated. Up to three Gcls that differ in their substrate specificity are present in genomes of *Lentilactobacillus* and *Limosilactobacillus* species. Of the strains used in this study, *Lm. reuteri* LTH5448 encodes for two Gcls and *Lm. reuteri* LTH2584 encodes for all three Gcls. Gcls are absent, however, in other heterofermentative lactobacilli, e.g. *Furfurilactobacillus*, *Apilactobacillus* and *Fructilactobacillus*, which implies that these enzymes contribute to the ecological fitness of lactobacilli only in specific habitats (Duar et al. 2017).

Lm. reuteri accumulates kokumi peptides during sourdough fermentation to concentrations that exceeds the threshold for kokumi activity; however, their concentration in sourdough bread remains to be determined (Zhao and Gänzle 2016; Yan et al. 2018). The synthesis of γ-glutamyl dipeptides and their contribution to food flavor differs substantially from the contribution of α-glutamyl dipeptides. α-Glu-dipeptides are released by proteolysis and their taste is dependent on the hydrophobicity of the second amino acid. α-Glutamyl dipeptides with residues of asp, glu or ser possess umami taste while α-glu-leu, α-glu-ile and α-glu-phe taste bitter (Zhao et al. 2016). In contrast, γ-glu-leu, γ-glu-ile and γ-glu-phe are kokumi-active. The present study on Gcl enzymes in lactobacilli extends prior knowledge on their accumulation in food fermentations by an initial characterization of the substrate specificity of three distinct Gcl enzymes. These presence and activity of these enzymes explains the strain specific differences observed in *Lm. reuteri* (Zhao and Gänzle 2016), however, the expression of the two or three enzymes present in a single strain remains to be determined. All three *Lm. reuteri* Gcls produced kokumi-active γ-glu-glu, γ-glu-met

and γ-glu-ala; Gcl1 and Gcl3 but not Gcl2 produced γ-glu-leu, γ-glu-ile and γ-glu-phe and only Gcl3 also produced γ-glu-gly, γ-glu-pro and γ-glu-ser. Of note, the pH during sourdough fermentation, pH 3.5 – 4.0, does not match the optimum pH of Gcl activity, however, the use of *gcl1*- and *gcl2*- deficient mutants of *Lm. reuteri* LTH5448 confirmed their activity during extended fermentation of type II sourdoughs (Yan et al. 2018). Because Gcl1 and Gcl3 produce a wider range of γ-glutamyl dipeptides when compared to Gcl1, the selection of fermentation cultures based on the substrate specificity of their Gcl enzymes may be a suitable tool to accumulate specific γ-glutamyl dipeptides and to improve food flavor through enhanced kokumi activity.

γ-Glu-val is a potent bioactive that reduces intestinal inflammation (Zhang et al. 2016). None of the three Gcls that were characterized in the present study synthesized γ-glu-val, however, other hydrophobic amino acids with unknown *in vivo* anti-inflammatory activity were substrates for Gcl1 and Gcl3. Whether or not *gcl* genes of *Lm. reuteri* regulate inflammatory processes in intestinal tract, or whether these relate to host-adaptation of specific sub-species of *Lm. reuteri* (Frese et al. 2011; Li et al. 2021) thus remains unknown.

In conclusion, the present study characterized three γ-glutamyl cysteine ligases from *Limosilactobacillus reuteri* that produce kokumi-active γ-glutamyl dipeptides. The differential presence of Gcls and GslAB in homofermentative and heterofermentative lactobacilli suggests that the synthesis of glutathione and γ-glutamyl dipeptides has a different contribution to the ecological fitness of these organisms. Gcl1, Gcl2 and Gcl3 from *Lm. reuteri* were similar with respect to their biochemical properties but differed with respect to their substrate specificities. These different substrate specificities may allow the selection of specific starter cultures for controlled formation of γ-glutamyl dipeptides in food. However, the cytoplasmic concentration of amino acids during

growth of lactobacilli in food fermentations differs from the concentration of amino acids in enzymatic reactions that were conducted in the present study, therefore, the contribution of the different Gcls to the synthesis of specific γ-glutamyl dipeptides in food remains to be elucidated.

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Ethical approval

This article does not describe contain any studies with human participants or animals performed by any of the authors.

Author contribution statement.

JX and MGG conceived and designed research. JX conducted experiments. JX and MGG analyzed data. JX and MGG wrote, read and approved the manuscript.

Conflict of Interest

JX declares that she has no conflict of interest.

MGG declares that he has no conflict of interest.

Data Availability Statement

All data generated or analysed during this study are included in this published article, or were deposited in public databases.

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Figure legends

Figure 1. Enzymatic reactions catalyzed by Gcl for synthesis of γ-glutamyl dipeptides.

Figure 2. SDS-PAGE analysis of glutamyl-cysteine ligases (Gcls) over-expressed in recombinant *Escherichia coli* BL21(DE3). Lane 1, molecular weight marker, the upper and lower arrows indicate proteins with 70 and 55kDa, respectively; lane 2: uninduced cell lysate of *E. coli* with pET28a$^+$; lane 3: induced cell lysate of *E. coli* with pET28a$^+$(*gcl1*); lane 4: purified Gcl1; lane 5: induced cell lysate of *E. coli* with pET28a$^+$(*gcl2*); lane 6: purified Gcl2; lane 7: Gcl3 inclusion body and cell wall preparation of induced *E. coli* with pET28a$^+$(*gcl3*); lane 8: renatured Gcl3).

Figure 3. Relative activity of Gcl1, Gcl1, and Gcl3 in presence of the Gcl inhibitor L-buthionine-sulfoximine (L-BSO). Enzymatic reactions were carried out in presence of 10.0mM Glu and 1.7 mM Cys at pH 8.0 and 37 °C.

Figure 4. Activity of Gcl1, Gcl2 and Gcl3 in presence of 13.3 mM of Mg^{2+} or 3.3 mM of all other metal ions. Enzymatic reactions were carried out in presence of 10.0 mM Glu and 1.7 mM Cys at pH 8.0 and 37 °C.

Figure 5. Relative activity of Gcl1, Gcl2 and Gcl3 at different pH values (**Panel A)** and at different temperatures (**Panel B).** Enzymatic reactions were carried out in presence of 10.0 mM Glu and 1.7 mM Cys; the effect of the pH was assayed at a temperature of 37 °C; the effect of the temperature was assayed at pH 9.0.

Figure 6. Kinetic characteristics of Gcl1, Gcl2 and Gcl3 with cysteine (**Panel A)** and glutamate (**Panel C)** as substrates. Enzymatic reactions were carried out at pH 9.0 and 43 °C and 10.0 mM Glu (**Panel A)** and 1.7 mM Cys (**Panel B).**

Figure 7. Phylogenetic tree of glutamyl cysteine ligases (Gcl) and the two-domain GSH synthetase (GshAB) from lactobacilli. The NCBI database was searched with Gcl1, Gcl2 or Gcl3 as query sequences with a BLAST cutoff of 25% amino acid identity. The results obtained with the three query sequences were combined and duplicate entries were removed; for each of Gcl1, Gcl2 or Gcl3, one sequence per protein (Gcl or GshAB) from the same species was kept to obtain a total of 221 protein sequences. Sequences that were more than 40% identical to Gcl1, Gcl2, or Gcl3 were annotated as Gcl1, Gcl2 or Gcl3, respectively; proteins sequences that included the two domains of Gsh synthetases were designated as GshAB; other proteins were designated as Gcl. Lactobacilli and their proteins are color coded as follows: Homofermentative and heterofermentative lactobacilli are coloured in black and orange, respectively. The five most frequent genera of lactobacilli are indicated by symbols; other genera are designated as "other lactobacilli". The protein type (Gcl1, Gcl2, Gcl3, other Gcl, or GshAB) is color coded on the outermost ring.

Table 1. Primers used in this study[a]

Primer (forward, F; reverse, R)	Sequence (5'-3')	Restriction site
gcl1 cloning, F	5'-ATGCA GGATCCATGTTTAGCAGAATTGG-3'	*Bam*HI
gcl1 cloning, R	5'-ATGCA CTCGAGCAATGTTAATTCTTTTCG-3'	*Xho*I
gcl2 cloning, F	5'-ATGCA GGATCCATGGGAACCGATTATGATC-3'	*Bam*HI
gcl2 cloning, R	5'-ATGCA CTCGAGCTTTTCCTGAAAATCCTG-3'	*Xho*I
gcl3 cloning, F	5'-ATGCA GGATCCATGTTAAGTAAATTTGGG-3'	*Bam*HI
gcl3 cloning, R	5'-ATGCA CTCGAGTTTTGCCGATAAATATTGC-3'	*Xho*I
gcl1, *gcl2* or *gcl3* sequencing, F1	5'-TAATACGACTCACTATAGG-3'	-
gcl1 sequencing, R1	5'-GTAAAATCACCGGTAAATTTGG-3'	-
gcl1 sequencing, F2	5'-CCAAATTTACCGGTGATTTTAC-3'	-
gcl2 sequencing, R1	5'-CCATGAAGTGCTTTAGTTCTG-3'	-
gcl2 sequencing, F2	5'-CAGAACTAAAGCACTTCATGG-3'	-
gcl3 sequencing, R1	5'-CGTGAATTTAGTTCCAAAACCG-3'	-
gcl3 sequencing, F2	5'-CGGTTTTGGAACTAAATTCACG-3'	-
gcl1, *gcl2* or *gcl3* sequencing, R2	5'-GCTAGTTATTGCTCAGCGG-3'	-

[a]Restriction sites are underlined.

Table 2. Pairwise amino acid identity (%) of Gcl domains from *Limosilactobacillus reuteri* and *Streptococcus agalacticae* (QDK30862.1)[a)]

	Gcl1	**Gcl2**	**Gcl3**
Gcl1 domain from *Lm. reuteri*	Q[b)]		
Gcl2 domain from *Lm. reuteri*	31.1	Q	
Gcl3 domain from *Lm. reuteri*	46.4	28.0	Q
Gcl domain from *S. agalacticae*	34.1	35.4	33.3

[a)] The 320 amino acid Gcl domains of the proteins was identified by InterPro software (www.ebi.ac.uk/interpro) and used as BLAST query on NCBI.

[b)] Q, query sequence with 100% identity.

Table 3 Qualitative analysis of γ-glutamyl peptides produced by Gcl1, Gcl2, or Gcl3 by LC-MS/MS.

γ-glutamyl dipeptides	Ion transition (m/z)	Retention time	Gcl1	Gcl2	Gcl3
γ-glutamyl glutamate	277.2/148.1	0.82	+	+	+
γ-glutamyl glutamine	276.2/147.1	0.95	+	+	+
γ-glutamyl methionine	279.2/150.1	0.95	+	+	+
γ-glutamyl cysteine	[a)]308.2/179.1	0.80	+	+	+
γ-glutamyl alanine	219.2/90.1	0.82	+	+	+
γ-glutamyl glycine	205.1/76.05	0.77	-	-	+
γ-glutamyl serine	235.2/106.1	0.75	±[b)]	-	+
γ-glutamyl leucine	261.2/132.1	6.66	+	-	+
γ-glutamyl isoleucine	261.2/132.1	6.14	+	-	+
γ-glutamyl phenylalanine	295.3/166.2	7.80	+	-	+
γ-glutamyl valine	247.2/118.1	n.d.[c)]	-	-	-
γ-glutamyl proline	245.2/116.2	0.95	±[b)]	-	+
γ-glutamyl aspartate	263.2/148.1	0.95	±[b)]	-	±
γ-glutamyl histidine	285.2/156.1	n.d.[c)]	-	-	-

[a)] γ-glutamyl cysteine was quantified after derivatization with iodoacetamide

[b)] The signal was less than 10 standard deviations higher than the baseline.

[c)] not detected, i.e. the signal was less than 3 standard deviations higher than the baseline.

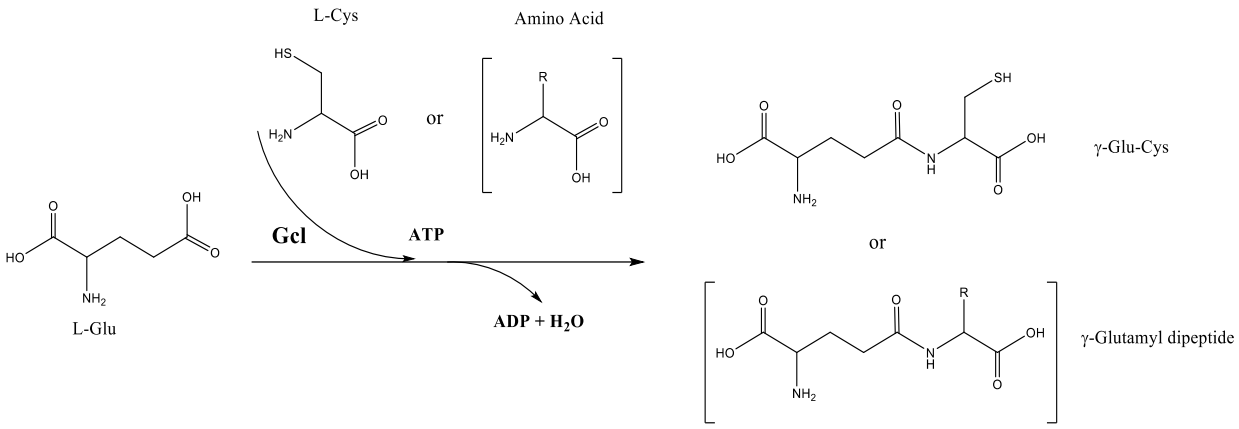

L-Cys
Amino Acid
or
L-Glu
Gcl
ATP
ADP + H2O
γ-Glu-Cys
or
γ-Glutamyl dipeptide

Figure 1.

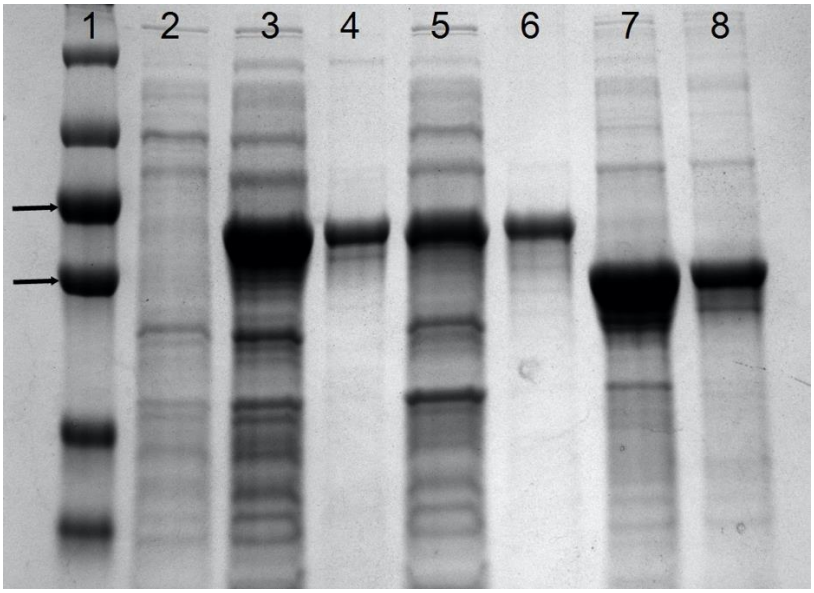


Figure 2.

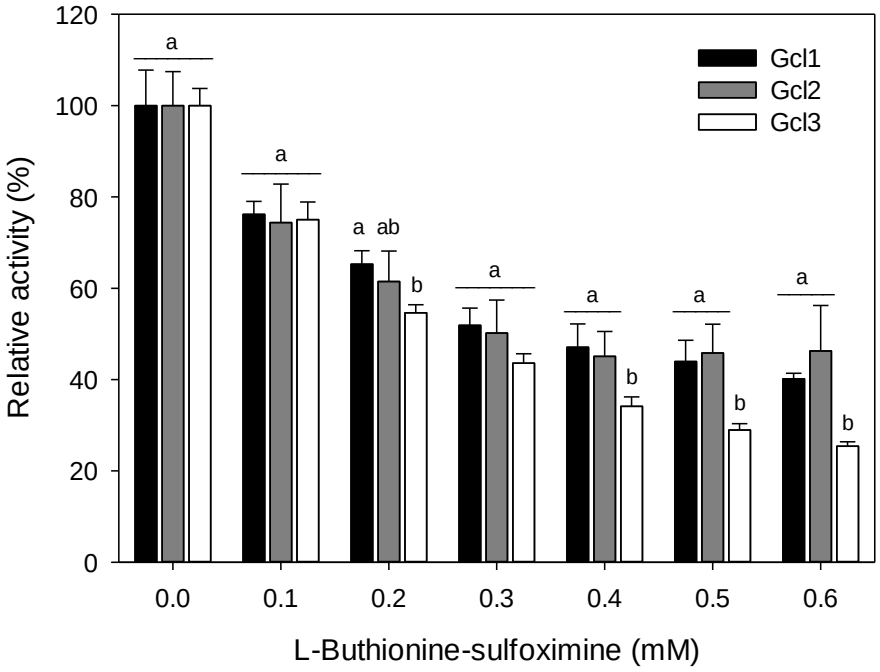

120
100
80
60
40
20
0
Relative activity (%)
a
a
a ab
b
a
a
b
a
b
a
b
Gcl1
Gcl2
Gcl3
0.0
0.1
0.2
0.3
0.4
0.5
0.6
L-Buthionine-sulfoximine (mM)

Figure 3.

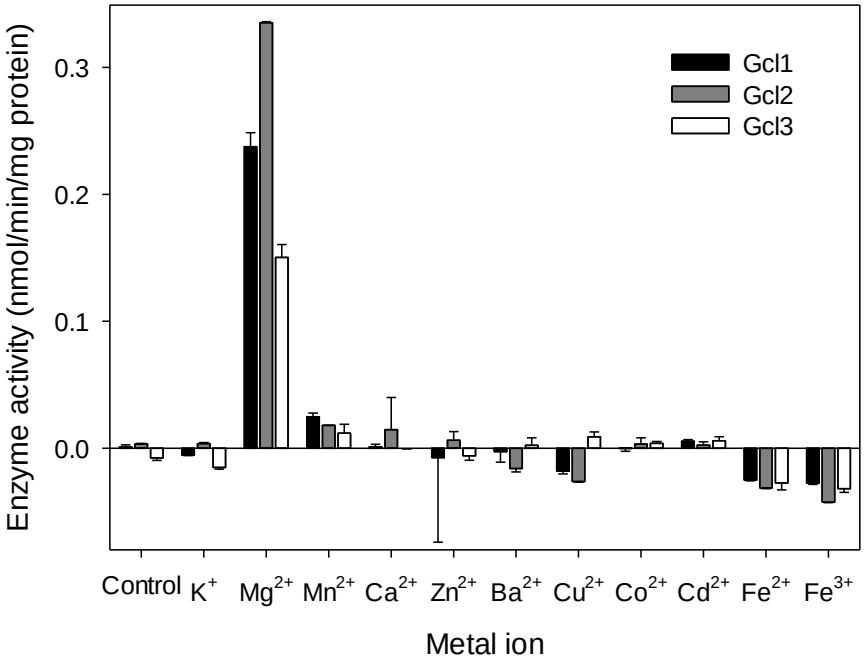

Enzyme activity (nmol/min/mg protein)
0.3
0.2
0.1
0.0
Gcl1
Gcl2
Gcl3
Control
K+
Mg2+
Mn2+
Ca2+
Zn2+
Ba2+
Cu2+
Co2+
Cd2+
Fe2+
Fe3+
Metal ion

Figure 4.

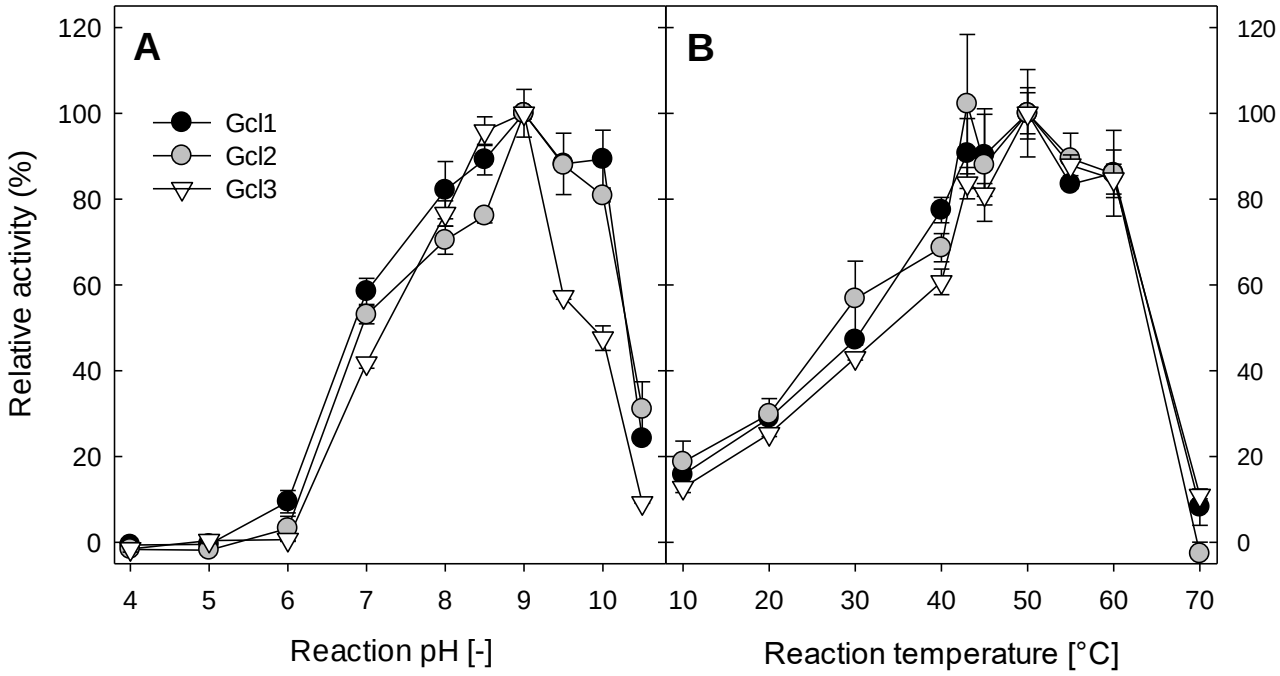

A
B
Gcl1
Gcl2
Gcl3
Relative activity (%)
Reaction pH [-]
Reaction temperature [°C]
0
20
40
60
80
100
120
4
5
6
7
8
9
10
10
20
30
40
50
60
70

Figure 5

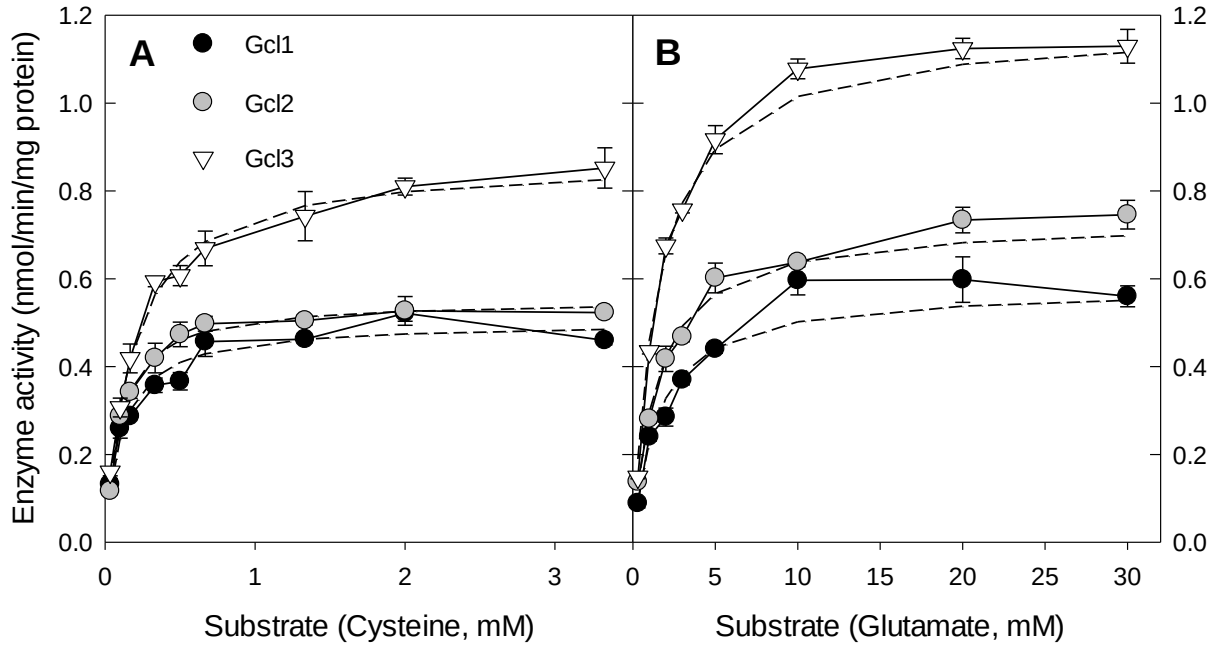
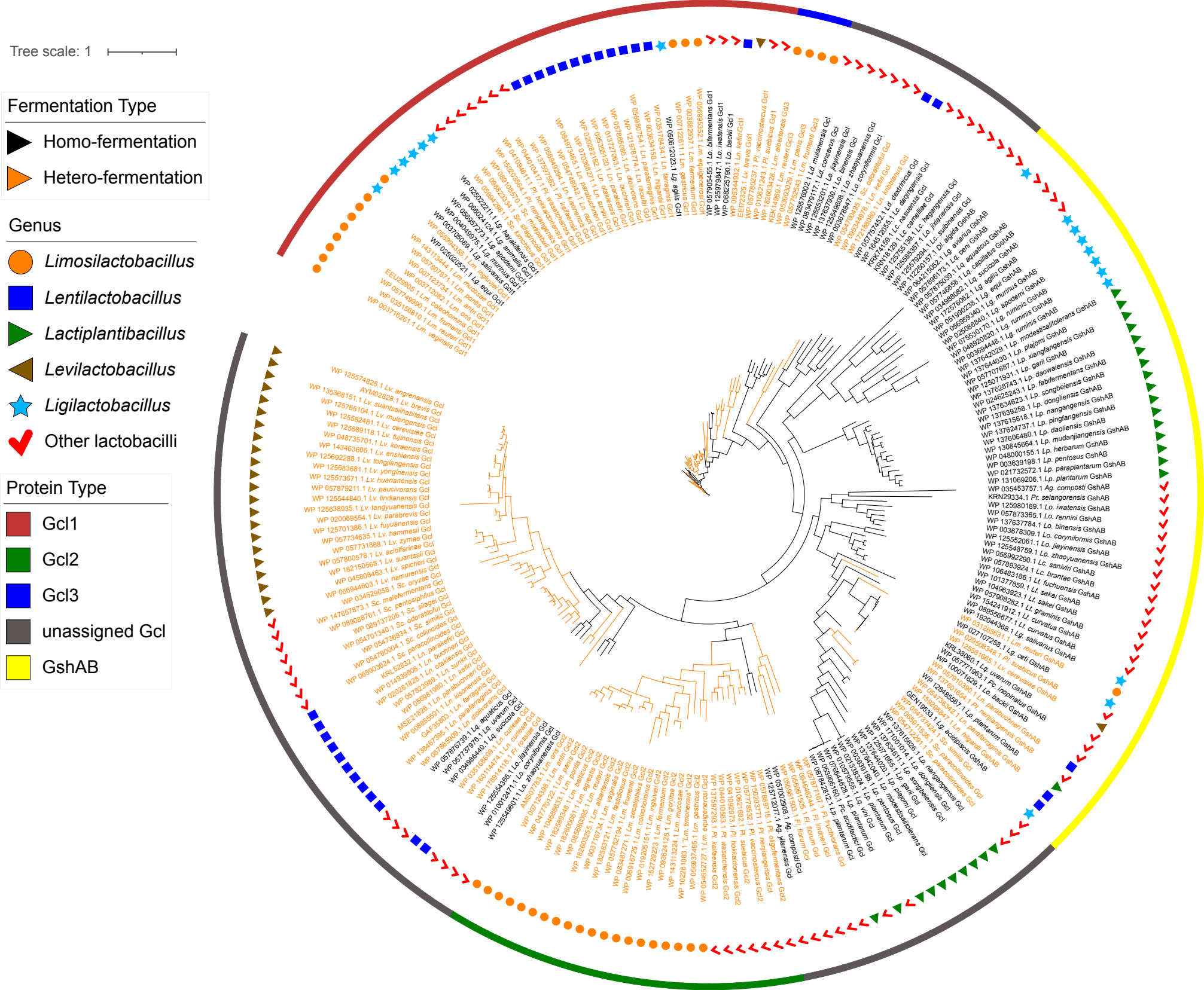

A
B
Gcl1
Gcl2
Gcl3
Enzyme activity (nmol/min/mg protein)
Substrate (Cysteine, mM)
Substrate (Glutamate, mM)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
1
2
3
5
10
15
20
25
30

Figure 6.


Tree scale: 1
Fermentation Type
Homo-fermentation
Hetero-fermentation
Genus
Limosilactobacillus
Lentilactobacillus
Lactiplantibacillus
Levilactobacillus
Ligilactobacillus
Other lactobacilli
Protein Type
Gcl1
Gcl2
Gcl3
unassigned Gcl
GshAB