Contribution of glutaminases to glutamine metabolism and acid resistance in *Lactobacillus reuteri* and other vertebrate host adapted lactobacilli

Qing Li[a], QianYing Tao[a], Jaunana S. Teixeira[a], Marcia Shu-Wei Su[a], Michael G. Gänzle[a,b*]

[a] University of Alberta, Dept. of Agricultural, Food and Nutritional Science, Edmonton, Alberta, Canada.

[b] Hubei University of Technology, College of Bioengineering and Food Science, Wuhan, Hubei, P.R. China

*Corresponding author footnote:

Michael Gänzle

University of Alberta,

Dept. of Agricultural, Food and Nutritional Science

4-10 Ag/For Centre,

Edmonton, AB,

Canada, T6G 2P5

Abstract

The bacterial conversion of glutamine to glutamate is catalyzed by glutamine-amidotransferases or glutaminases. Glutamine deamination contributes to the formation of the bioactive metabolites glutamate, γ-aminobutyrate (GABA) and γ-glutamyl peptides, and to acid resistance. This study aimed to investigate the distribution of glutaminase(s) in lactobacilli, and to evaluate their contribution in *L. reuteri* to amino acid metabolism and acid resistance. Phylogenetic analysis of the glutaminases *gls1, gls2* and *gls3* in the genus *Lactobacillus* demonstrated that glutaminase is exclusively present in host-adapted species of lactobacilli. The disruption *gls1*, *gls2* and *gls3* in *L. reuteri* 100-23 had only a limited effect on the conversion of glutamine to glutamate, GABA, or γ-glutamyl peptides in sourdough. The disruption of all glutaminases in *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* but not disruption of *gls2* and *gls3* eliminated the protective effect of glutamine on the survival of the strain at pH 2.5. Glutamine also enhanced acid resistance of *L. reuteri* 100-23Δ*gadB* and *L. taiwanensis* 107q, strains without glutamate decarboxylase activity. Taken together, the study demonstrates that glutaminases of lactobacilli do not contribute substantially to glutamine metabolism but enhance acid resistance. Their exclusive presence in host-adapted lactobacilli provides an additional link between the adaptation of lactobacilli to specific habitats and their functionality when used as probiotics and starter cultures.

Keywords: *Lactobacillus reuteri*, Glutaminase, intestinal lactobacilli, Acid resistance, sourdough

1. Introduction

The fermentation of sourdough confers characteristic taste and flavor to bread and steamed bread. Proteolysis and conversion of amino acids during sourdough fermentation provide taste compounds and precursor compounds for flavor volatiles (Thiele et al., 2002). Glutamine is the most abundant amino acid in wheat and rye proteins; cereal proteases and microbial peptidases release glutamine during sourdough fermentation (Thiele et al., 2002; Gänzle et al., 2008). Microbial metabolism converts glutamine to glutamate, which imparts the umami taste of bread (Vermeulen *et al.*, 2007; Stromeck et al., 2011; Zhao et al., 2015). Glutamate is a substrate for further conversion to γ-aminobutyrate (GABA) (Coda et al., 2010; Siragusa et al., 2007; Su et al., 2011). Dietary GABA may regulate immune responses and mediate anti-hypertensive effects (Oh and Oh, 2003; Inoue et al., 2003). Glutamate is also a substrate for the production of γ-glutamyl peptides (Zhao and Gänzle, 2016), agonists of calcium-sensing receptor proteins in taste cells that enhance umami, sweet and salty tastes. This influence on taste perception has been termed "kokumi" (Maruyama et al., 2012; Ohsu et al., 2010), and described as mouthfulness, thickness, and continuity of taste (Ueda et al., 1994).

Glutamine-amidotransferases or glutaminases [EC 3.5.1.2] catalyse the bacterial conversion of glutamine to glutamate (Strohmeier et al., 2006). *Lactobacillus reuteri* 100-23 harbours three glutaminases, one of these genes is part of a glutamine/glutamate operon that also codes for glutamate decarboxylase (GadB) and two putative glutamate/GABA antiporters (GadC) (Su et al., 2011; Teixeira et al., 2014). The

61 contribution of these glutaminases on the formation of glutamine metabolites in
62 sourdough, however, remains unclear.

63 The extended fermentation time of type II sourdoughs imposes acid stress on
64 lactobacilli (Vogel et al., 1999; De Vuyst et al., 2009). Glutamate decarboxylation
65 increases the acid resistance of lactobacilli including *L. reuteri* through intracellular
66 decarboxylation, which consumes protons, and electrogenic antiport of glutamate and
67 GABA (Su et al., 2011; Teixeira et al., 2014). Glutamate-mediated acid resistance
68 increases the ecological fitness of *L. reuteri* in the rodent forestomach, and in type II
69 sourdough fermentations (Krumbeck et al., 2016; Lin and Gänzle, 2014). Glutamine
70 deamination also increased acid resistance of *Escherichia coli* and *L. reuteri* (Brown et
71 al., 2008; Lu et al., 2013; Teixeira et al., 2014). In *L. reuteri* and *Brucella microti*
72 CCM4915 but not in *E. coli*, glutaminase, glutamate decarboxylase and a
73 glutamate/GABA antiporter are arranged in a single operon (Teixeira et al., 2014;
74 Freddi et al., 2017, Pennacchietti et al., 2018). It was the aim of this study to investigate
75 the distribution of glutaminase in lactobacilli, to evaluate the contribution of
76 glutaminase in *L. reuteri* to amino acid metabolism in sourdough, and to determine
77 whether glutaminase contributes to acid resistance in lactobacilli. The study generated
78 *L. reuteri* 100-23 Δ*gls1*Δ*gls2*Δ*gls3*, a derivative with deletion of all three *gls* in the
79 genome of *L. reuteri* 100-23.

80 **2. Materials and Methods**

81 2.1. Strains, plasmids and growth conditions

Table 1 shows bacterial strains and plasmids used in this study. *L. reuteri* 100-23, *L. taiwanensis* 107q, *L. johnsonii* 117a and *L. acidophilus* FUA3066 were cultivated in modified deMan-Rogosa-Sharpe (mMRS, Gänzle and Vogel, 2003) medium at 37 °C. *E. coli* JM 109 (Promega, Nepean, Canada) was cultured in Luria-Bertani (LB) medium at 37 °C. The frozen stock cultures were inoculated on agar plates; single colonies were inoculated in 1 ml broth, subcultured with 1 % inoculum in broth. LB medium with ampicillin (100 mg / L) or erythromycin (500 mg / L) was used for antibiotic-resistant *E. coli* selection. Erythromycin-resistant *L. reuteri* was selected by adding erythromycin (5 mg / L) into mMRS medium.

2.2. Phylogenetic analysis of glutaminase in lactobacilli

Protein sequences of glutaminases in genomes of all *Lactobacillus* species were retrieved from the National Center for Biotechnology Information database using BlastP (Altschul et al., 1997) and the sequences of three glutaminases in *L. reuteri* 100-23 as query sequences. Protein sequences were retrieved with a cut-off of 30% amino acid identity; sequences were discarded if an identical sequence from a second strain in the same species was available. The 21 remaining sequences were aligned by MEGA7 (Kumar et al., 2016) using ClustalW method. The phylogenetic analysis of glutaminases was conducted by the Maximum Likelihood method based on the JTT matrix-based mode (Jones et al., 1992) and the bootstrap support values were calculated from 500 replicates by MEGA7.

102 2.3. Generation and confirmation of *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3*.

103 The genome of *L. reuteri* 100-23 encodes for three glutaminases, namely Gls1, Gls2,
104 and Gls3 (Teixeira et al., 2014). A triple mutant *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* was
105 constructed by truncating *gls1*, *gls2*, and *gls3* using pJRS233 (Perez-Casal et al., 2005)
106 according to a double crossover mutagenesis method (Su et al., 2008). Table 1 lists
107 plasmids used for the construction of mutant strains and Table 2 lists primers. PCR with
108 primers *gls2*-KO1-BamHI and *gls2*-KO2-XbaI and *gls2*-KO3-XbaI and *gls2*-KO4-PstI
109 amplified 5' and 3' flanking fragments of *gls2*, respectively, using genomic DNA from
110 *L. reuteri* 100-23 as a template. The 5' and 3'-flanking fragments of *gls2* were ligated
111 to pGEMTeasy vector (Promega) to generate pGLS2-AB. The DNA fragment in
112 pGLS2-AB was cut with *Bam*HI and *Sal*I, purified and ligated into pJRS233 carrying
113 an erythromycin resistance gene as a selective marker to generate pGLS2-KO-AB. The
114 resulting plasmid pGLS2-KO-AB was electro-transformed in *L. reuteri* cells.
115 Transformants were incubated in mMRS-erm broth at 42-44 °C or 80 generations to
116 select for single crossover mutants. These single crossover mutants were subsequently
117 incubated for 100 generations in mMRS broth at 37 °C. Replica plating on mMRS and
118 mMRS-erm agar identified erythromycin sensitive derivatives that lost the plasmid by
119 a double-crossover event; *L. reuteri* 100-23Δ*gls2* were identified by PCR with primers
120 *gls2*-5F and *gls2*-6R. The deletion was verified by amplification of the truncated *gls2*
121 with primers *gls2*-F and *gls2*-R. Subsequently, *L. reuteri* 100-23 Δ*gls2*Δ*gls3* was
122 generated by interrupting *gls3* in *L. reuteri* 100-23Δ*gls2* with the same protocol and

123 plasmids and primers shown in Table 1 and 2. *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* was
124 constructed by disruption of *gls1* in *L. reuteri* 100-23Δ*gls2*Δ*gls3*. PCR analysis and
125 DNA sequencing (MacrogenUSA, Rockville, MD) verified the deletion regions using
126 primers *gls1*-F and *gls1*-R, *gls2*-F and *gls2*-R, and *gls3*-F and *gls3*-R.

127 2.4. Accumulation of amino acids during buffer fermentation

128 Overnight cultures of *L. reuteri* 100-23, *L. reuteri* Δ*gadB*, and *L. reuteri*
129 Δ*gls1*Δ*gls2*Δ*gls3* were harvested by centrifugation and washed twice with 50 mmol/L
130 sodium acetate buffer (pH 4.5). Cells were re-suspended in 50 mM Sodium acetate
131 buffer or buffers supplemented with 10 mmol / L glutamine or 10 mmol / L glutamate.
132 Buffer without inoculum served as control. Samples were collected after 0 and 8 hours
133 of incubation for monitoring bacterial survival and quantification of amino acids.
134 Buffer fermentations were carried out in three biological replicates.

135 2.5. Accumulation of amino acids during sourdough fermentation

136 Sourdoughs were fermented with *L. reuteri* 100-23, *L. reuteri* 100-23Δ*gadB* or *L.*
137 *reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* respectively to determine cell counts, pH and amino
138 acid accumulation during growth in sourdough. Two gram of wheat flour, 2 ml of
139 sterilized tap water were incubated with an initial cell count of $1 \pm 0.5 \times 10^7$ CFU / g.
140 Samples were collected after 0, 12, 24 and 48 hours of incubation. Sourdough
141 fermentations were carried out in three biological replicates.

142 2.6. Quantification of amino acids by high performance liquid chromatography (HPLC)

143 Amino acids were quantified by HPLC after derivatization with o-phthaldialdehyde
144 (Sedgwick et al., 2011). Buffer samples were centrifuged to remove cells; the
145 supernatant was mixed with 5% w / v trichloroacetic acid and β-aminobutyric acid,
146 which served as internal standard. The mixture was centrifuged and the supernatant was
147 then derivatized with ortho-phthalaldehyde (1 vol) with addition of saturated potassium
148 borate (5 vol) (Sedgwick et al., 2011). Sourdough samples were lyophilized and
149 extracted with water at an extraction ratio of 1:6 (w / v); the supernatant was mixed
150 with 5 % w / v trichloroacetic acid and β-aminobutyric acid (Stromeck et al., 2011).
151 The mixture was then derivatized with o-phthaldialdehyde described above.

152 2.7. Synthesis of γ-glutamyl dipeptides during buffer fermentation and sourdough
153 fermentation

154 Buffer fermentations were performed with *L. reuteri* 100-23 and *L. reuteri* 100-
155 23Δ*gls1*Δ*gls2*Δ*gls3* as described (Zhao and Gänzle, 2016) with modifications. Cells
156 from overnight cultures of three strains were washed twice with autoclaved tap water,
157 and re-suspended in 20 mmol / L phosphate buffer (pH 6.5) containing 5 g / L of maltose,
158 10 mmol / L of glutamine, and 10 mmol / L of lysine, glutamate, leucine, isoleucine,
159 phenylalanine or valine as glutamate acceptors. Buffers were incubated at 37 °C for 24
160 h. Samples were collected before and incubation for LC-MS/MS analysis of γ-glutamyl
161 dipeptides in the culture supernatant. Buffer inoculated with strains and maltose but
162 without the addition of amino acids, and uninoculated buffers served as controls. The
163 cell count and pH were monitored on each sample. Buffer fermentations were carried

164 out in two independent experiments, and samples were analyzed in duplicate.

165 Sourdoughs were fermented as described above with *L. reuteri* 100-23 or *L. reuteri*
166 100-23Δ*gls1*Δ*gls2*Δ*gls3*. Sourdough samples were collected at 0 h and 48 h and
167 lyophilized. Then 0.5g lyophilized sourdough was mixed with1 ml of 0.1 % formic acid
168 and the mixture was incubated at 25 °C for 1 h with 250 rpm shaking. The mixture was
169 diluted at a ratio of 1: 10 into 30 % methanol and pellets in the dilution was collected
170 by centrifuge. The supernatant was collected and filtered for LC-MS/MS analysis.

171 2.8. Quantification of γ-glutamyl dipeptides by LC-MS/MS

172 γ-Glutamyl dipeptides were quantified by LC-MS/MS as described (Zhao and Gänzle,
173 2016). In short, peptides were separated on a 1200 series HPLC unit with diode array
174 detector (Agilent Technologies, Palo Alto, CA, USA) connected to a 4000 Q TRAP LC-
175 MS/MS system (MDS SCIEX, Applied Biosystems, Streetsville, ON, Canada). The
176 mobile phase consisted of 0.1 % formic acid in Milli-Q water (solvent A) and 0.1 %
177 formic acid in acetonitrile (solvent B). Samples were eluted from an Express C18 HPLC
178 column (2.7 μm, 150 mm × 2.1 mm, Phenomenex, Torrance, CA, USA) at a flow of 0.2
179 ml / min with the following gradient: 0 min, 95 % A; 10 min, 75 % A; 15 min, 0 % A;
180 followed by re-equilibration with 95 % A. LC-MS/MS parameters for quantitation of
181 the six kokumi peptides are shown in Table 3. External calibration standards (0.1−100
182 μg / L) of γ-glutamyl dipeptides were prepared at 30 % (v / v) methanol in 0.1 %
183 aqueous formic acid.

184 2.9. Survival of lactobacilli at pH 2.5 and 3.5

185 To determine the survival of lactobacilli at acid pH in presence or absence of glutamine,
186 *L. reuteri* 100-23, *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3*, *L. reuteri* 100-23Δ*gadB*, *L.*
187 *acidophilus* FUA3066, *L. johnsonii* 117a, and *L. taiwanensis* 107q were incubated at
188 pH 2.5 or 3.5 with the addition of 10 mmol / L glutamine or glutamate. Cells from
189 overnight cultures were washed in 50 mmol / L Na_2HPO_4 buffer (pH 7.0) and re-
190 suspended in 50 mmol / L potassium phosphate buffer (pH 2.5) or in 50 mmol / L lactate
191 buffer (pH 3.5). Viable plate counts were determined by surface plating on mMRS agar.
192 Experiments were performed in three biological replicates.

193 2.10. Statistical analysis.

194 Data analysis was performed with IBM SPSS statistics 23, using one-way or two-way
195 analysis of variance (ANOVA). A *P*-value of ≤ 0.05 was considered statistically
196 significant.

197 2.11. Nucleotide sequence accession numbers.

198 The genome sequences of *L. reuteri* 100-23 were obtained from the National Center for
199 Biotechnology Information databases (GenBank: AAPZ02000001.1 and
200 AAPZ02000002.1). Nucleotide sequences and annotations were retrieved from
201 GenBank with accession numbers AAPZ02000001.1:333174-334094,
202 AAPZ02000001.1:1455778-1456692, and AAPZ02000002.1:548219-549139 for
203 genes *gls1*, *gls2*, and *gls3*, respectively. The sequences of the truncated *gls1*, *gls2*, and

gls3 in *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* were deposited with accession number MN147878, MN147879, and MN147880, respectively.

3. Results.

3.1. Phylogenetic analysis of glutaminases in *Lactobacillus* species

A phylogenetic analysis determined the frequency and distribution of glutaminases in the genus *Lactobacillus* (Figure 1A). Gls2 of *L. reuteri* 100-23 is least similar to other glutaminases and has no homologues in other lactobacilli. Three and two genomes of other *L. reuteri* group species harboured homologues of Gls1 and Gls3, respectively. Ten genomes of *L. delbrueckii* group organisms, as well as *L. lindneri* (*L. fructivorans* group) and *L. aviarius* (*L. salivarius* group) harboured putative glutaminases that clustered separately from *L. reuteri* enzymes. All lactobacilli with glutaminases are adapted to animal hosts; with the exception of *L. lindneri*, glutaminases were present in organisms that are adapted to the intestine of vertebrate animals (Duar et al., 2017). We additionally analysed whether glutaminases in lactobacilli are part of operons that also include GadB and / or GadC. The genetic loci of glutaminases of lactobacilli that are part of an operon including GadC and / or GadB shown in Figure 1B. *L. aviarius spp. aviarius* is the only other *Lactobacillus* spp. that harbours an operon with glutaminase, GadB and GadC. *L. lindneri* encodes for a GadC/Gls operon. In other lactobacilli with glutaminase, GadC was present *in trans; L. antri* and *L. oris* encoded for a GadB /GadC operon distant from Gls (data not shown). None of the *L. delbrueckii* group species with glutaminase encoded for GadB.

225 3.2. Glutamine metabolism of *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* in sourdough.

226 To test the role of glutaminases of *L. reuteri* in glutamine metabolism in sourdough, the
227 concentrations of glutamine, glutamate and GABA were measured during wheat
228 sourdough fermentation with *L. reuteri* 100-23, 100-23Δ*gls1*Δ*gls2*Δ*gls3*, and
229 100-23Δ*gadB* respectively (Fig. 3). The growth of strains and the pH of sourdough were
230 also monitored (Supplement Fig. 1A). The growth of three strains in sourdough was
231 identical throughout fermentation, the cell counts increased to the maximum after 12 h
232 and maintained at ~10 log CFU g^{-1} during the following 24 h fermentation, indicating
233 that the growth of strains during sourdough fermentation was not influenced by the
234 truncation of *gadB* or three *gls*.

235 The disruption of *gls* genes or *gadB* had no influence on the concentration of (glutamine
236 + glutamate + GABA) after 48 h of fermentation. Glutamine accumulated in chemically
237 acidified dough but its concentration remained low in all sourdoughs. *L. reuteri* 100-23
238 converted glutamine to GABA and accumulated highest level of GABA after 48 h of
239 fermentation. The deletion of *gadB* resulted in glutamate accumulation and low GABA
240 concentrations. GABA concentration in sourdough fermented with *L. reuteri*
241 100-23Δ*gls1*Δ*gls2*Δ*gls3* was higher than the concentration in sourdough fermented
242 with *L. reuteri* 100-23Δ*gadB*.

243 3.3. Synthesis of γ-glutamyl dipeptides in buffer and sourdoughs

Glutamyl-cysteine ligases in *L. reuteri* LTH5448 synthesize γ-Glu-Ile and γ-Glu-Cys but deletion of both glutamyl-cysteine ligases did not fully eliminate synthesis of γ-glutamyl peptides in *L. reuteri* LTH5448 (Yan et al., 2018). Bacterial glutaminases catalyze transglutamination to produce γ-glutamyl peptides (Nandakumar et al., 2003; Tomita et al., 1988), therefore, the γ-glutamyltransferase activity of glutaminase in *L. reuteri* 100-23 was assessed by monitoring the concentration of γ-glutamyl dipeptides after incubation of the strain and its mutants in buffers (Figure 3) and in sourdough (Table 5). The deletion of *gls* decreased the synthesis of γ-Glu-Glu in buffers, however, the formation of other γ-glutamyl peptides was not affected (Fig. 3). Deletion of glutaminases in *L. reuteri* did not affect the synthesis of γ-glutamyl dipeptides in sourdoughs (Table 5).

3.4. Effects of glutamine and glutamate on acid resistance of *L. reuteri* 100-23

The survival of *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* and *L. reuteri* 100-23Δ*gadB* was compared with that of the wild type *L. reuteri* 100-23 in phosphate buffer (pH 2.5) and lactate buffer (pH 3.5). Amino acids supplementation did not influence survival at pH 3.5 and *L. reuteri* 100-23Δ*gadB* was the most sensitive strain (Figure 4). During incubation at pH 2.5, glutamate supplementation protected the wild type strain and *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* but not *L. reuteri* 100-23Δ*gadB*. The effect of glutamine supplementation on survival of *L. reuteri* 100-23 was comparable to the effect of glutamate. Glutamine supplementation increased survival of *L. reuteri* 100-23Δ*gadB* when compared to supplementation with glutamate, however, glutamine did

not improve or decreased survival of *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* when compared to glutamate supplementation (Figure 4).

Previous studies suggested that overexpression of *gls1*, *gls2* and *gadB* compensates the disruption of *gls3* in *L. reuteri* 100-23 (Zhang, 2011). To assess the role of the three glutaminases in acid resistance, *L. reuteri* 100-23, 100-23Δ*gls3*, *L. reuteri* 100-23Δ*gls2*Δ*gls3* and 100-23Δ*gls1*Δ*gls2*Δ*gls3* were incubated in phosphate buffer of phosphate buffer containing glutamate or glutamine (Figure 5). Glutamine protected all strains except *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* against acid challenge, indicating that Gls2 or Gls1 compensate for the loss of Gls3, or Gls2 and Gsl3.

3.5. Survival of host-adapted lactobacilli at pH 2.5 and pH 3.5

To confirm the contribution of glutamine metabolism to the acid resistance of other host-adapted lactobacilli, survival of *L. johnsonii* 117a, *L. taiwanensis* 107q and one strain of *L. acidophilus* FUA3066 at pH 2.5 or 3.5 was determined. All three organisms are host-adapted organisms in the *L. delbrueckii* group; the genome sequences of *L. johnsonii* 117a and *L. taiwanensis* 107q contain a glutaminase gene (Lin et al., 2018) but none of the available genome sequences of *L. acidophilus* encompasses a glutaminase. The absence of glutaminases in *L. acidophilus* FUA3066 was confirmed with primers targeting the conserved regions of glutaminases of in *L. taiwanensis, L. johnsonii,* and *L. gasseri*, and in *L. crispatus* (Table 4). At pH 2.5, supplementation of glutamine and glutamate improved survival of *L. taiwanensis* 107q but amino acid

supplementation did not improve survival of *L. johnsonii* 117a or *L. acidophilus* (Figure 6). Addition of glutamate or glutamine did not improve the survival of any of the strains at pH 3.5.

4. Discussion

This study investigated the contribution of glutaminase to glutamine metabolism in sourdough and to acid resistance of lactobacilli. Phylogenetic analysis of glutaminase sequences indicates glutaminase of *Lactobacillus* species is exclusively present in host-adapted lactobacilli. The deletion of glutaminases in *L. reuteri* did not influence glutamine and glutamate metabolism in sourdough but decreased the acid resistance of *L. reuteri* at pH 2.5.

During sourdough fermentation, the most abundant amino acid released from wheat and rye proteins is glutamine. Microbial conversion of glutamine yields glutamate, an umami tastant, GABA, or kokumi-active γ-glutamyl peptides. Glutaminase hydrolyzes γ-amino group of L-glutamine to produce L-glutamic acid (Nandakumar et al., 2003; Prusiner et al., 1976). Glutaminase activity in *L. reuteri* KCTC3594 was described as a salt- and thermotolerant enzyme with activity in the range of pH 5.0 – 11.0 (Jeon et al., 2009; Jeon et al., 2009a). The molecular weight of the partially purified enzymes (50 – 70 kDa), however, does not match the molecular weight of Gls1 (33.47 kDa), Gls2 (33.25 kDa) or Gsl3 (33.15 kDa) (Jeon et al., 2009, Teixeira et al., 2014). This suggests the presence of multiple enzymes in *L. reuteri* with activity on glutamine. Accordingly, the disruption of all three glutaminases in *L. reuteri* 100-23 had no effect on the

306 glutamine metabolism by *L. reuteri* in sourdough (Fig. 2). In buffer, the truncation of
307 glutaminases reduced the synthesis of γ-glutamyl-glutamate (Fig. 3) and GABA (data
308 not shown), suggesting a reduced rate of glutamine conversion to glutamate.

309 Glutamine amidotransferases also catalyze the removal of the ammonia group from
310 glutamine and subsequently transfer of ammonia to a specific substrate (Trotta et al.,
311 1973). A wide range of biosynthetic enzymes, including carbamoyl-phosphate synthase,
312 pyridoxal 5'-phosphate synthase, and guanosine monophosphate synthase, contain
313 glutamine amidotransferase subunits with glutaminase activity (Thoden et al., 1998;
314 Zalkin et al., 1985; Strohmeier et al., 2006). Those genes that are encoded in the genome
315 of *L. reuteri* 100-23 that relate to glutamine conversion to glutamate are glutamine
316 amidotransferases including a GMP synthase [EDX43269.1], an asparagine synthase
317 [EDX43329.1], a peptidase C26 [EDX43401.1], carbamoyl-phosphate synthase
318 [EDX41777.1], and an isomerizing glutamine-fructose-6-phosphate aminotransferase
319 [EDX41955.1]. The glutaminase activity of these biosynthetic enzymes likely accounts
320 for glutaminase conversion in *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3*, for glutaminase
321 activities of *Lactobacillus rhamnosus* (Weingand-Ziadé et al., 2003) and *Lactobacillus*
322 *sanfranciscensis* DSM20451 (Vermeulen et al., 2007), strains which harbour glutamine
323 amidotransferases but not glutaminase. The γ-glutamyltranspeptidase from *Bacillus*
324 *subtilis* also exhibits glutaminase activities at pH 8.0-8.5, but is absent in genome of *L.*
325 *reuteri* 100-23 (Morelli et al., 2013; Su et al., 2011). In conclusion, glutaminases are
326 accessory enzymes that are present only in few lactobacilli and do not make a

substantial contribution to metabolic phenotypes.

The deamination of glutamine contributes to acid resistance of bacteria. Glutaminase Ybas in *E. coli* is active at acidic pH and protects *E. coli* at pH 2.5 via the release of ammonia (Lu et al., 2013). A contribution of glutaminase to acid resistance of *L. reuteri* is documented by the loss of protective effect of glutamine on acid resistance of *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* (this study), and by the demonstration that glutamine remains protective for *L. reuteri* 100-23Δ*gadB* (this study, Teixeira et al., 2014).

The glutaminase *gls3* in *L. reuteri* 100-23 is part of an operon that also includes *gadB* and *gadC*; a comparable operon structure is found in *Brucella microti* (Lu et al., 2013; Freddi et al., 2017). The *gls-gadB/C* operon is prevalent in Gram-negative bacteria but rarely found in Gram-positive bacteria (Pennacchietti et al., 2018). Among *Lactobacillus* species, the *gls-gadB/C* operon is present only in *L. reuteri* and *L. aviarius* subsp. *aviarius*; *L. lindneri* harboured a *gls-gadC* operon. The presence of glutaminase and glutamate decarboxylase in enteric bacteria and rodent-lineage strains of *L. reuteri* strongly indicates their role to protect bacteria against acid stress in gut or the transit through the stomach (Pennacchietti et al., 2018; Su et al., 2012; Beasley et al., 2015, Krumbeck et al., 2016, this study). Comparison of the glutamine-mediated acid resistance and the operon structure in lactobacilli and *E. coli* suggests that glutamine-mediated acid resistance requires GadC and Gls and that the system is functional even if the two genes are not part of the same operon. This is further supported by the lack of an acid resistance phenotype in *L. reuteri* 100-23Δ*gls3 and*

100-23Δ*gls2*Δ*gls3*, where the remaining glutaminase genes, *gls1* and *gls2*, or *gls1*, are located distant from the GadC/GadB operon (this study, Teixeira et al., 2014). *L. taiwanensis* 107q maintains glutaminase activity without GadB or a GadC. Accordingly, glutamine addition increased acid resistance of this strain much less when compared to *L. reuteri* 100-23. Because glutamine and glutamate are alternative substrates for this acid resistance mechanism, deletion of one or the other has a modest but significant impact on the acid resistance of *L. reuteri* 100-23 *in situ* and *in vivo* (Lin and Gänzle, 2014; Krumbeck et al., 2016; this study).

Type II sourdough microbiota are derived from vertebrate-adapted lactobacilli in the *L. delbrueckii* and *L. reuteri* groups (Gänzle and Zheng, 2018; Hammes and Hertel, 2006; Su et al., 2012). Acid resistance is a physiological trait that increases competitiveness in type II sourdoughs as well as intestinal ecosystems (Lin and Gänzle, 2014; Gänzle and Zheng, 2018; Krumbeck et al., 2016; Zheng et al., 2015). This study demonstrated that glutaminase is exclusively present in lactobacilli adapted to vertebrate intestinal ecosystems. Among these, the glutaminase based acid resistance is predominantly but not exclusively maintained by *L. reuteri*, *L. taiwanensis,* and *L. johnsonii* that represent rodent forestomach microbiota (Frese et al., 2011; Duar et al., 2017; Lin et al., 2018). The genomes of *L. taiwanensis* 107q and *L. johnsonii* 117a encode for glutaminase but not for GadB activities or glutamine or GadC (this study; Lin et al., 2018), suggesting that lactobacilli alternatively maintain glutamine or glutamate based mechanisms of acid resistance. Utilizing the concept of “lifestyle” in sourdough ecology thus helps to

explain how specific metabolic traits in lactobacilli contribute to the competitiveness and bread quality during sourdough fermentation (Gänzle and Zheng, 2018).

In conclusion, this study demonstrated the exclusive presence of glutaminase in host-adapted lactobacilli and the contribution of glutaminase to acid resistance of *L. reuteri* 100-23. Glutaminase-mediated acid resistance is thus a "lifestyle-associated" metabolic trait that is shared with Gram-negative pathogens. The accumulation of GABA without glutaminase implies that an alternative pathway of glutamine deamination exists in *L. reuteri* 100-23 to hydrolyze glutamine and produce glutamate as the precursor of GABA.

Acknowledgements

Sabine Schlicht is acknowledged for contributions in assembly of plasmids pGLS2-AB and pGLS3-AB. Oy Karl Fazer Ab and Alberta Wheat are acknowledged for financial support. Qing Li and Michael Gänzle acknowledge support by the China Scholarship Council and the Canada Research Chairs program, respectively.

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Figure legends.

Figure 1. Analysis of glutaminases in the genus *Lactobacillus.* **Panel A**. Phylogenetic analysis of glutaminases in the genus *Lactobacillus*. Sequences in all genomes of the genus *Lactobacillus* were identified by BLASTp using *gls1*, *gls2* or *gls3* as query sequences. Identical sequences from the same species were discarded. Phylogenetic tree was constructed based on 21 glutaminase sequences from 12 *Lactobacillus* species by using the Maximum Likelihood method based on the JTT matrix-based model (Jones, 1992) and the bootstrap support values were calculated from 500 replicates by MEGA7 (Kumar et al., 2016). Bootstrap values are shown if they are higher than 50. Branch lengths were measured in the number of substitutions per site. Roman numerals designate the phylogenetic groups: *L. delbrueckii* (I)*, L. reuteri* (II)*, L. salivarius* (III) and *L. fructivorans* (IV). **Panel B**. Genetic loci of those *gls* sequences that are adjacent to *gadB* or *gadC* in genus *Lactobacillus*. *Brucella microti* CCM4915 is used for comparison. Nucleotide sequences and annotations were retrieved from GenBank with accession numbers AAPZ02000002.1: 548219-549139, LVKF01000063.1: 29436-30365, JQBT01000033.1: 133694-134626 and CP001579.1: 322403-323356 for glutaminases in *L. reuteri* 100-23*, L. aviarus* UMNLAv4*, L. lindneri* DSM20690 and *Brucella microti* CCM4915, respectively. Gene name: *gls* (glutaminase), *gadB* (glutamate decarboxylase), *gadC* (glutamate/GABA antiporter).

Figure 2. Concentration of glutamine (A), glutamate (B) and γ-aminobutyric acid (C) during sourdough fermentation with *L. reuteri* 100-23 (▲), *L. reuteri* Δ*gls1*Δ*gls2*Δ*gls3*

561 (Δ) or *L. reuteri* Δ*gadB* (■) over 48 h. Chemically acidified dough fermented for 48 h
562 served as control (□). Symbols indicate means ± standard deviation of three biological
563 replicates. Values for chemically acidified sourdough or for sourdoughs fermented with
564 isogenic mutant derivatives of *L. reuteri* 100-23 are marked with an asterisk if they
565 differ significantly (P<0.05) from values for sourdoughs fermented with *L. reuteri* 100-
566 23.

567 **Figure 3.** Concentration of γ-glutamyl dipeptides in buffers fermented with *L. reuteri*
568 100-23 (black columns), *L. reuteri* 10-23Δ*gls1*Δ*gls2*Δ*gls3* (gray columns) after 24h
569 fermentation. Symbols indicate means ± standard deviation from two biological
570 replicates analyzed in duplicate. Values for different peptides that do not share a
571 common uppercase superscript differ significantly (P<0.05). Values for the same
572 peptide that do not share a common lowercase superscript differ significantly between
573 samples obtained after incubation with *L. reuteri* 100-23 and *L. reuteri* 10-
574 23Δ*gls1*Δ*gls2*Δ*gls3* (P<0.05).

575 **Figure 4.** Acid resistance of *L. reuteri* 100-23 (black symbols), *L. reuteri*
576 *gls1*Δ*gls2*Δ*gls3* (grey symbols) or *L. reuteri* Δ*gadB* (open symbols) in phosphate buffer
577 (pH 2.5, A), in phosphate buffer with 10mM glutamate (B), or in phosphate buffer with
578 10 mM glutamine (C). Acid resistance of *L. reuteri* 100-23 (black symbols), *L. reuteri*
579 *gls1*Δ*gls2*Δ*gls3* (grey symbols) or *L. reuteri* Δ*gadB* (open symbols) in lactate buffer
580 (pH 3.5, D), in phosphate buffer with 10mM glutamate (E), or in phosphate buffer with
581 10 mM glutamine (F). Symbols indicate means ± standard deviation from biological

582 replicates. Values for different strains in the same buffer at the same incubation time do
583 not share a common lowercase superscript differ significantly (P<0.05). Values
584 obtained in buffers with glutamate or glutamine are marked with an asterisk if they
585 differ significantly (P<0.05) from values obtained at the same incubation time with the
586 same strain in control buffer. Values for the same strain in buffers with glutamine are
587 marked with a plus sign if they differ significantly (P<0.05) from values obtained with
588 the same strain at the same incubation time in buffer with glutamate (P<0.05).

589 **Figure 5.** Acid resistance of *L. reuteri* 100-23, 100-23Δ*gls3*, *L. reuteri* 100-
590 23Δ*gls2*Δ*gls3* and 100-23Δ*gls1*Δ*gls2*Δ*gls3* in phosphate buffer (pH 2.5, black bars), in
591 phosphate buffer with 10mM glutamate (light grey bars), or in phosphate buffer with
592 10 mM glutamine (dark grey bars). Data represent means ± standard deviation from
593 biological replicates. Values for different strains in the same buffer at the same
594 incubation time do not share a common lowercase superscript differ significantly
595 (P<0.05). Values obtained with the same strain in different buffers differ significantly
596 (P<0.05) if they do not share a common superscript.

597 **Figure 6.** Acid resistance of *L. johnsonii* 117a (A), *L. taiwanensis* 107q (B) or *L.*
598 *acidophilus* (C) in phosphate buffer (pH 2.5, black symbols), in phosphate buffer with
599 10mM glutamate (open symbols), or in phosphate buffer with 10 mM glutamine (grey
600 symbols). Acid resistance of *L. johnsonii* 117a (D), *L. taiwanensis* 107q (E) or *L.*
601 *acidophilus* (F) in lactate buffer (pH 3.5, black symbols), in phosphate buffer with 10
602 mmol / L glutamate (open symbols), or in phosphate buffer with 10 mmol / L glutamine

603 (grey symbols). Symbols indicate means ± standard deviation from triplicates. Values
604 for the same strain in different buffers at the same incubation time do not share a
605 common lowercase superscript differ significantly ($P<0.05$).
606

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmid	Genotype	Source or reference
	Strains	
Lactobacillus reuteri 100-23	Rodent isolate; wild type strain	Wesney et. al.
Lactobacillus johnsonii 117a	Rodent isolate; wild type strain	Lin et. al., 2018
Lactobacillus taiwanensis 107q	Rodent isolate; wild type strain	Lin et. al., 2018
Lactobacillus acidophilus FUA3066	Isolate from commercial probiotic culture	This study
L. reuteri Δ*gls3*	*Lactobacillus reuteri* 100-23 derivative with deletion of *gls3*	This study
L. reuteri Δ*gls2*Δ*gls3*	*Lactobacillus reuteri* 100-23 derivative with deletions of *gls2* and *gls3*	This study
L. reuteri Δ*gls1*Δ*gls2*Δ*gls3*	*Lactobacillus reuteri* 100-23 derivative with deletions of *gls1, gls2,* and *gls3*	This study
L. reuteri Δ*gadB*	*Lactobacillus reuteri* 100-23 derivative with deletions of *gadB*	Su et al., 2011
Escherichia coli JM109	Cloning host for pGEMTeasy- and pJRS233-deviative plasmids	Promega
	Plasmids	
pGEMTeasy	Cloning vector used in *E. coli*; 3.0 kb; Amp^r	Promega
pGLS1-A	pGEMTeasy containing 1 kb of the DNA sequence upstream of *gls1*; 4.0 kb; Amp^r	This study
pGLS1-B	pGEMTeasy containing 1 kb of the DNA sequence downstream of *gls1*; 4.0 kb; Amp^r	This study
pGLS1-AB	pGEMTeasy containing upstream and downstream sequences of *gls1*; 5.0 kb; Amp^r	This study
pJRS233	Shuttle vector used in the hosts *E. coli* and *Lactobacillus reuteri* 100-23, Erm^r	Perez-Casal et. al., 2005
pGLS1-KO-AB	pJRS233 containing 2.0 kb of the flanking sequences of *gls1*; Erm^r	This study
pGLS2-A	pGEMTeasy containing 1 kb of the DNA sequence upstream of *gls2*; 4.0 kb; Amp^r	This study
pGLS2-B	pGEMTeasy containing 1 kb of the DNA sequence downstream of *gls2*; 4.0 kb; Amp^r	This study
pGLS2-AB	pGEMTeasy containing upstream and downstream sequences of *gls2*; 5.0 kb; Amp^r	This study
pGLS2-KO-AB	pJRS233 containing 2.0 kb of the flanking sequences of *gls2*; Erm^r	This study
pGLS3-A	pGEMTeasy containing 1 kb of the DNA sequence upstream of *gls3*; 4.0 kb; Amp^r	This study
pGLS3-B	pGEMTeasy containing 1 kb of the DNA sequence downstream of *gls3*; 4.0 kb; Amp^r	This study
pGLS3-AB	pGEMTeasy containing upstream and downstream sequences of *gls3*; 5.0 kb; Amp^r	This study
pGLS3-KO-AB	pJRS233 containing 2.0 kb of the flanking sequences of *gls3*; Erm^r	This study

Table 2. Primers used in the construction of *L. reuteri* 100-23 Δ*gls1*Δ*gls2*Δ*gls3*.

Distupted gene	Primers	Primer sequences (5'-3')
gls1	*gls1*-KO1-PstI	AACTGCAGGGGATTGTAACTTGAAATTAAC
	gls1-KO2-BglII	GAAGATCTCATTCTTGAATTGCGTCATTAAG
	gls1-KO3-BglII	GAAGATCT AGGTACTAGTTGCAAATATTCGC
	gls1-KO4-BamHI	CGGGATCC GATATTCAGCAGTCGAAAG
	gls1-5F	GCCAAATATCTGCTGATCG
	gls1-6R	AACAGCGTTTGTTCCAA
	gls1-F	TGGCTGATTCCAGTCACATTAG
	gls1-R	GAGTGGGAAGTAAGGGACAAAG
gls2	*gls2*-KO1-BamHI	CGGGATCCTTGCCGATGCATTAAC
	gls2-KO2-XbaI	GCTCTAGACTATTGCTCTAATTTTTGCATCGT
	gls2-KO3-XbaI	GCTCTAGATTAGAATTAGTAGTTTAATAAAAGCG
	gls2-KO4-PstI	AACTGCAGGGAAACGCAGATGAGAG1263-1297
	gls2-5F	AGAGCGGGGTATTTCG
	gls2-6R	GCTGGTTGGGTAAAAGTT
	gls2-F	ACAATACTCAAGCCGACCTAAC
	gls2-R	CTATACCCAGCGTGTGAAGAAA
gls3	*gls3*-KO1-PstI	AACTGCAGAAAAGCTTGGACAACCC
	gls3-KO2-EcoRI	GGAATTCTTATTTAAGATCCAAAGTAATCACCTC
	gls3-KO3-EcoRI	GGAATTC TTTCAGTACTAATAATTAAGGTCCAA
	gls3-KO4-BamHI	CGGGATCCGCATGTGCTGAAAATTG
	gls3-5F	CCTTTATCAACCATCAGCT
	gls3-6R	AGCTGGTGTGCTACTTT
	gls3-F	GATTCCAGCAAAGCCAAACC
	gls3-R	GCTGAAGATACCACCACCATTA

Table 3. LC-MS/MS parameters for the determination of γ-glutamyl dipeptides in water-soluble extracts of sourdough.

Peptide	Transition	Retention time (min)	DP	CE
γ-Glutamyl glutamate	277.3 / 84.1	1.89	50	40
γ-Glutamyl cysteine	251.2 / 122.2	1.95	40	17
γ-Glutamyl valine	247.2 / 72.1	2.78	50	35
γ-Glutamyl isoleucine	261.2 / 132.5	8.40	50	19
γ-Glutamyl leucine	261.2 / 132.5	9.30	50	19

Table 4. Primers used to confirm the absence of *gls* in *L. acidophilus* FUA3066

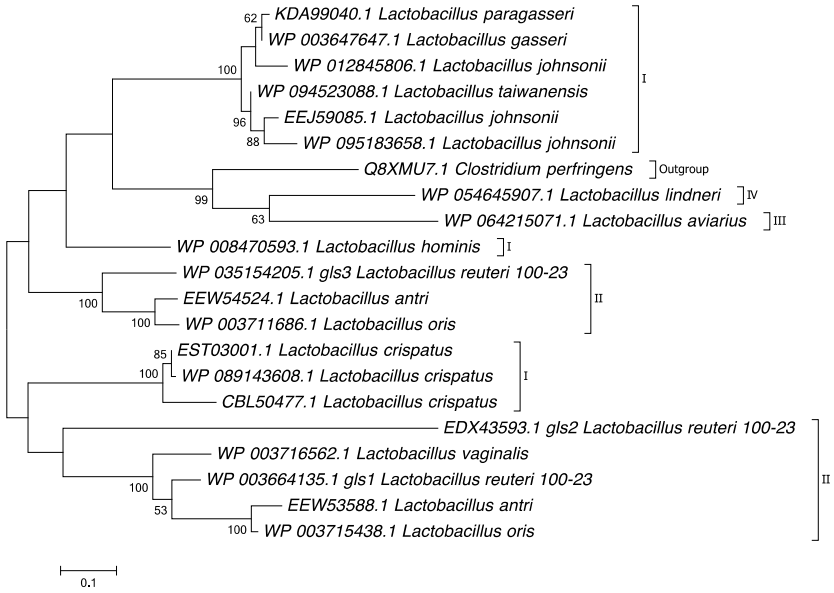
Targeted strains	**Primers**	**Primer sequences (**5'-3')
L. gasseri, L. taiwanensis, L. johnsonii	Forward	CATGGGACAACAAACGCATTAT
	Reverse	GACATAAGACCACCACCAACA
L. crispatus	Forward	ATGATGGTAACGGCACGTAG
	Reverse	CACCTCCAACACCACTCTTAG
L. antri, L. oris	Forward	CCCGCACATTACCCTCAATAAT
	Reverse	AAATCAGTCGTTCCTGATCCC

Table 5. Concentration of γ-glutamyl dipeptides in wheat sourdough fermentations

Dipeptides	**γ-Glu-Glu**	**γ-Glu-Cys**	**γ-Glu-Ile**	**γ-Glu-Leu**	**γ-Glu-Val**
			0 h		
L. reuteri 100-23	3.39 ± 1.46^{a}	1.46 ± 0.13^{b}	0.30 ± 0.05^{a}	0.51 ± 0.18^{a}	0.56 ± 0.05^{a}
100-23Δ*gls1*Δ*gls2*Δ*gls3*	4.56 ± 0.74^{a}	1.40 ± 0.17^{b}	0.27 ± 0.07^{a}	0.41 ± 0.11^{a}	0.58 ± 0.05^{a}
Chemically acidified	3.34 ± 1.06^{a}	2.11 ± 0.35^{a}	0.35 ± 0.10^{a}	0.47 ± 0.14^{a}	0.60 ± 0.08^{a}
			48 h		
L. reuteri 100-23	8.08 ± 2.19^{a}	1.07 ± 0.44^{a}	5.27 ± 0.09^{a}	0.91 ± 0.38^{a}	0.62 ± 0.10^{a}
100-23Δ*gls1*Δ*gls2*Δ*gls3*	5.74 ± 0.86^{ab}	1.28 ± 0.41^{a}	5.62 ± 0.58^{a}	1.08 ± 0.40^{a}	0.63 ± 0.07^{a}
Chemically acidified	3.37 ± 0.39^{b}	0.92 ± 0.17^{a}	0.58 ± 0.23^{b}	0.96 ± 0.16^{a}	0.58 ± 0.04^{a}

Concentration of γ-glutamyl dipeptides in sourdoughs fermented with different strains at the same incubation time do not share a common lowercase superscript differ significantly (P<0.05).

A


KDA99040.1 Lactobacillus paragasseri
WP 003647647.1 Lactobacillus gasseri
WP 012845806.1 Lactobacillus johnsonii
WP 094523088.1 Lactobacillus taiwanensis
EEJ59085.1 Lactobacillus johnsonii
WP 095183658.1 Lactobacillus johnsonii
Q8XMU7.1 Clostridium perfringens
Outgroup
WP 054645907.1 Lactobacillus lindneri
IV
WP 064215071.1 Lactobacillus aviarius
III
WP 008470593.1 Lactobacillus hominis
I
WP 035154205.1 gls3 Lactobacillus reuteri 100-23
EEW54524.1 Lactobacillus antri
WP 003711686.1 Lactobacillus oris
II
EST03001.1 Lactobacillus crispatus
WP 089143608.1 Lactobacillus crispatus
CBL50477.1 Lactobacillus crispatus
I
EDX43593.1 gls2 Lactobacillus reuteri 100-23
WP 003716562.1 Lactobacillus vaginalis
WP 003664135.1 gls1 Lactobacillus reuteri 100-23
EEW53588.1 Lactobacillus antri
WP 003715438.1 Lactobacillus oris
II
0.1

B

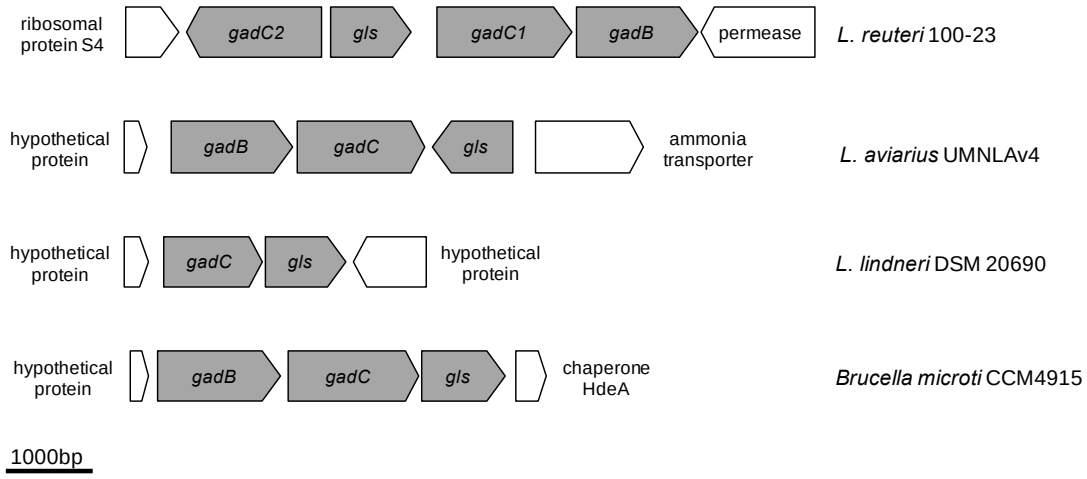

ribosomal protein S4
gadC2
gls
gadC1
gadB
permease
L. reuteri 100-23
hypothetical protein
gadB
gadC
gls
ammonia transporter
L. aviarius UMNLAv4
hypothetical protein
gadC
gls
hypothetical protein
L. lindneri DSM 20690
hypothetical protein
gadB
gadC
gls
chaperone HdeA
Brucella microti CCM4915
1000bp

Figure 1.

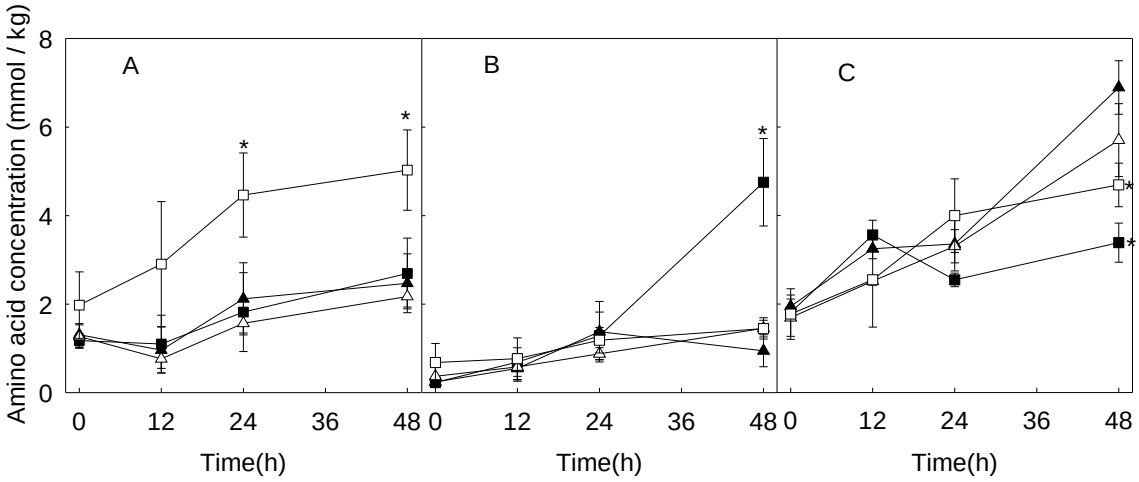

Amino acid concentration (mmol / kg)
A
B
C
Time(h)
Time(h)
Time(h)
0
2
4
6
8
0
12
24
36
48
*

Figure 2.

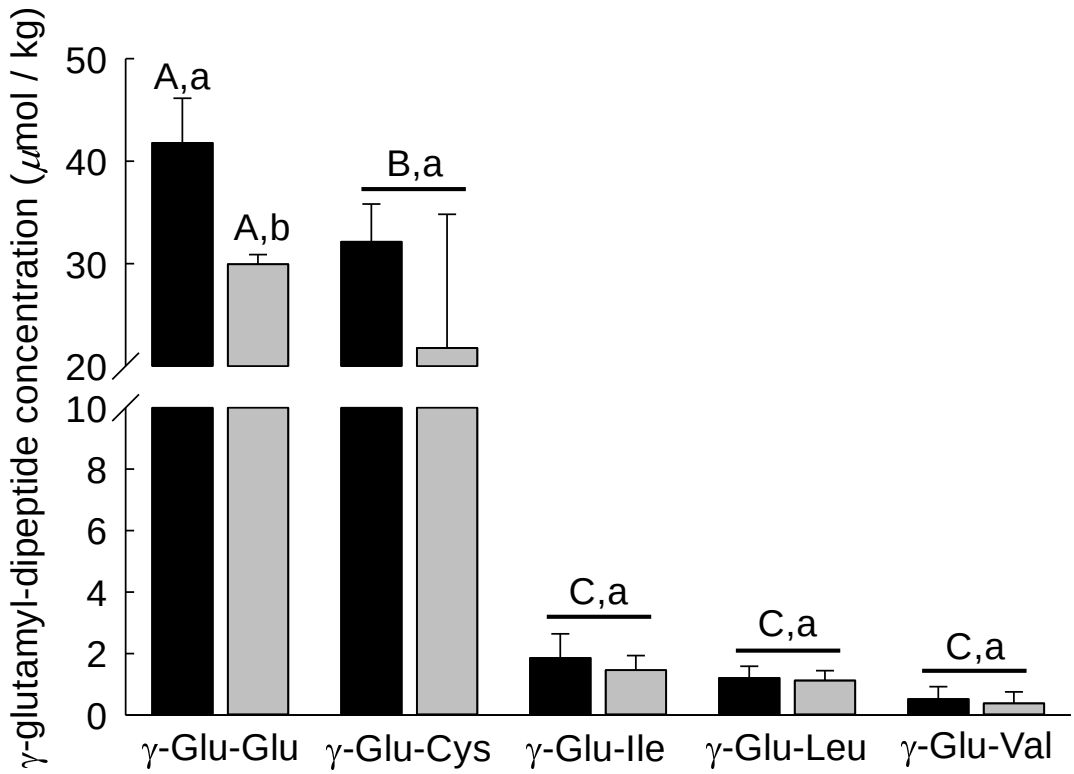

γ-glutamyl-dipeptide concentration (μmol / kg)
50
40
30
20
10
8
6
4
2
0
A,a
A,b
B,a
C,a
C,a
C,a
γ-Glu-Glu
γ-Glu-Cys
γ-Glu-Ile
γ-Glu-Leu
γ-Glu-Val

Figure 3.

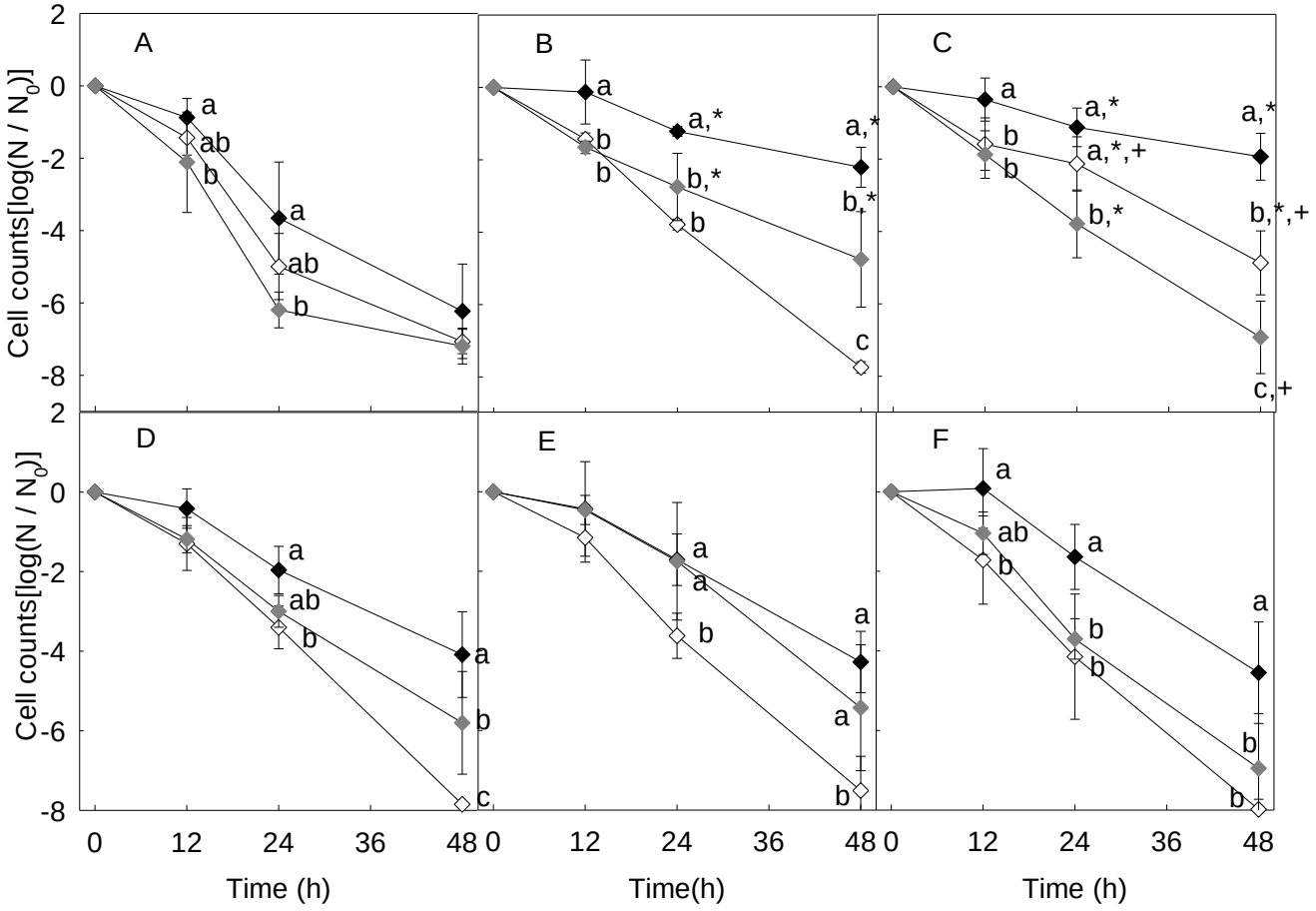

A
B
C
D
E
F
Cell counts[log(N / N0)]
Time (h)
Time(h)
0
12
24
36
48
2
0
-2
-4
-6
-8
a
ab
b
a,*
b,*
c
a,*,+
b,*,+
c,+

Figure 4.

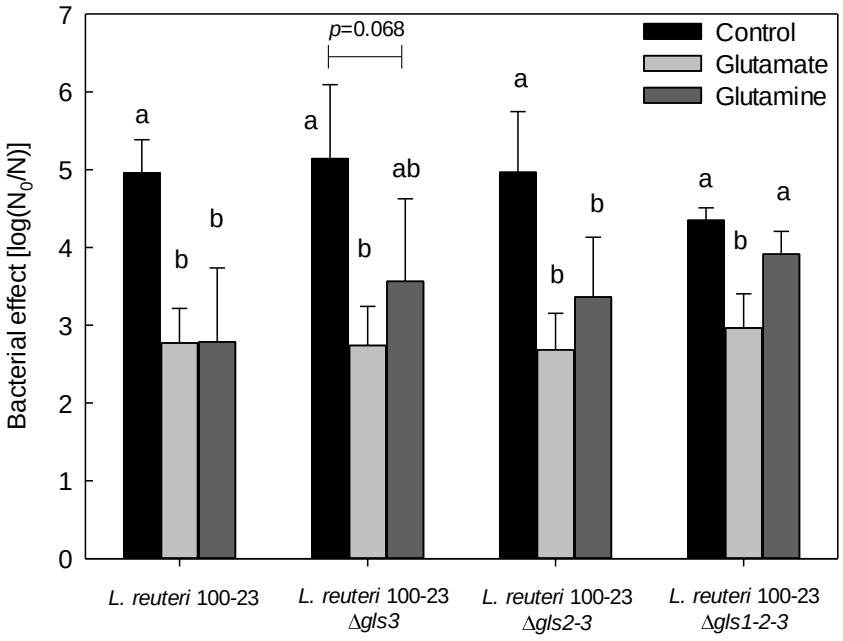

Bacterial effect [log(N0/N)]
7
6
5
4
3
2
1
0
p=0.068
Control
Glutamate
Glutamine
a
b
b
a
b
ab
a
b
b
a
b
a
L. reuteri 100-23
L. reuteri 100-23 Δgls3
L. reuteri 100-23 Δgls2-3
L. reuteri 100-23 Δgls1-2-3

Figure 5.

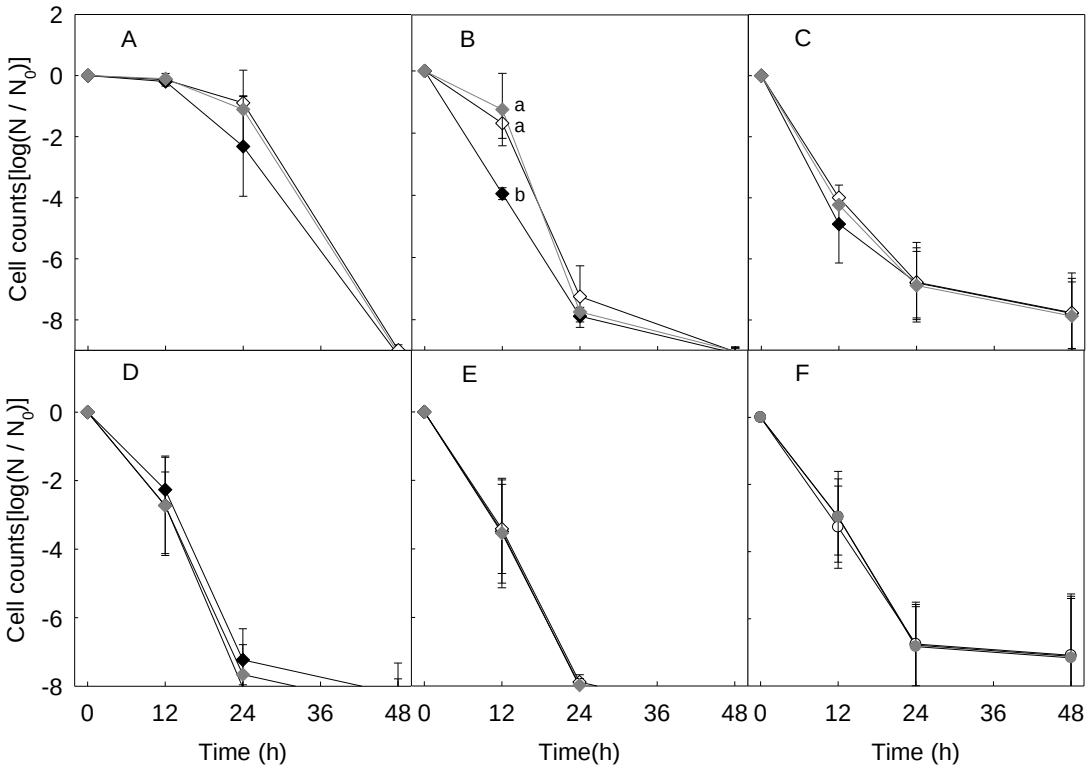

A
B
C
D
E
F
a
a
b
Cell counts[log(N / N0)]
Cell counts[log(N / N0)]
2
0
-2
-4
-6
-8
0
12
24
36
48
Time (h)
Time(h)

Figure 6.

Characterization of glutaminase, an enzyme contributing to acid resistance in *Lactobacillus reuteri* and other vertebrate host adapted lactobacilli

Qing Li[a], QianYing Tao[a], Jaunana S. Teixeira[a], Marcia Shu-Wei Su[a], Michael G. Gänzle[a,b*]

[a] University of Alberta, Dept. of Agricultural, Food and Nutritional Science, Edmonton, Alberta, Canada.

Online supplementary material.

Figure S1. Cell counts (A) and pH (B) a during sourdough fermentation with *L. reuteri* 100-23 (▲), *L. reuteri* Δ*gls1*Δ*gls2*Δ*gls3* (Δ) or *L. reuteri* Δ*gadB* (■) over 48 h.

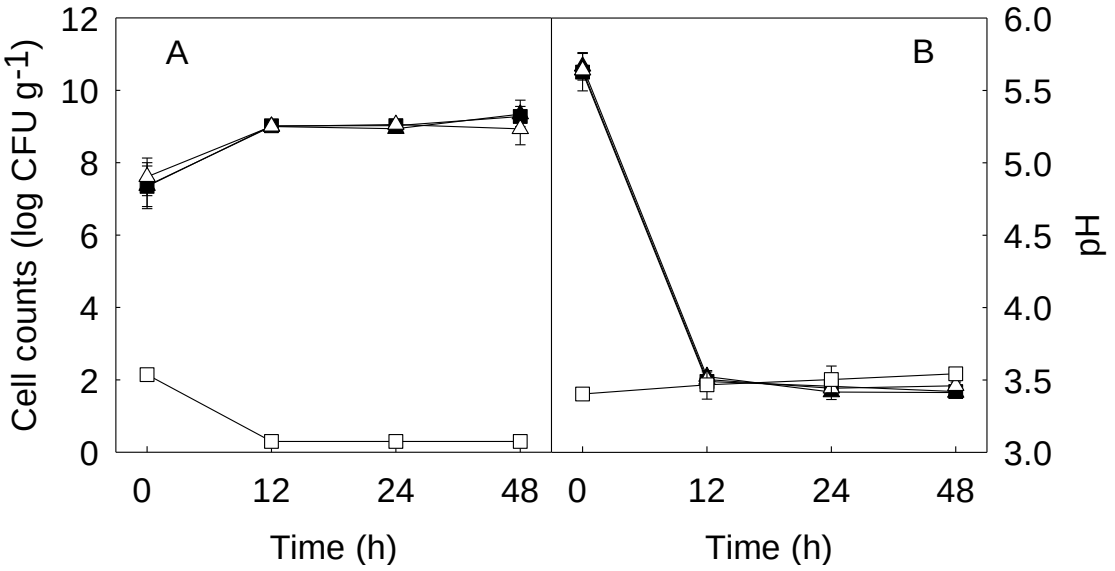


Figure S1. Cell counts (A) and pH (B) a during sourdough fermentation with *L. reuteri* 100-23 (▲), *L. reuteri* Δ*gls1*Δ*gls2*Δ*gls3* (Δ) or *L. reuteri* Δ*gadB* (■) over 48 h. Chemically acidified dough fermented for 48 h served as control (□). Symbols indicate means ± standard deviation of three biological replicates.