Glutamine, glutamate, and arginine-based acid resistance in *Lactobacillus reuteri*

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Abstract

This study aimed to determine whether glutamine deamidation improves acid resistance of *Lactobacillus reuteri*, and to assess whether arginine, glutamine, and glutamate-mediated acid resistance are redundant or complementary mechanisms of acid resistance. Three putative glutaminase genes, *gls1, gls2,* and *gls3,* were identified in *L. reuteri* 100-23. All three genes were expressed during growth in mMRS and wheat sourdough. *L. reuteri* consistently over-expressed *gls3* and the glutamate decarboxylase *gadB*. *L. reuteri* 100-23Δ*gadB* over-expressed *gls3* and the arginine deiminase gene *adi*. Analysis of the survival of *L. reuteri* in acidic conditions revealed that arginine conversion is effective at pH of 3.5 while glutamine or glutamate conversion were effective at pH of 2.5. Arginine conversion increased the pH_{in} but not $\Delta\Psi$; glutamate decarboxylation had only a minor effect on the pH_{in} but increased the $\Delta\Psi$. This study demonstrates that glutamine deamidation increases the acid resistance of *L. reuteri* independent of glutamate decarboxylase activity. Arginine and glutamine / glutamate conversions confer resistance to lactate at pH of 3.5 and phosphate at pH of 2.5, respectively. Knowledge of *L. reuteri*'s acid resistance improves the understanding of the adaptation of *L. reuteri* to intestinal ecosystems, and facilitates the selection of probiotic and starter cultures.

Keywords: *Lactobacillus reuteri* 100-23, glutaminase, arginine deiminase, glutamate decarboxylase, acid resistance

1. Introduction

Lactobacillus reuteri inhabits the intestinal tract of humans and animals; stable populations are found in the upper intestine of rodents, pigs, and poultry (Walter, 2008). *L. reuteri* also prevails in Type II sourdough fermentations, carried out at elevated fermentation temperatures with long incubation times (Vogel et al., 1999). Both ecosystems impose acid stress. Type II sourdough fermentations expose *L. reuteri* to lactic acid at pH of 3.5 for several days (Vogel et al., 1999; Stromeck et al., 2011). In the rodent forestomach, digesta remain for up to 20 h and are acidified to a pH of 3.5 to 4 by lactic metabolism (Gärtner, 2001; Ward and Coates, 1987). Subsequent gastric passage further acidifies digesta by gastric secretions of HCl (Ward and Coates, 1987).

Lactobacilli respond to acid stress by differential expression of stress proteins, and by up-regulation of metabolic pathways that contribute to pH homeostasis (De Angelis and Gobbetti, 2004, Konings, 2002). The multitude of acid resistance mechanisms implies that *L. reuteri* is well equipped to withstand acidic environments (Figure 1). Metabolic activities of *L. reuteri* that increase acid resistance also contribute to its ecological fitness in sourdough and in the rodent intestine (Rollan et al., 2003; Kaditzky et al., 2008; Walter et al., 2008; Su et al., 2011).

The role of arginine and glutamate metabolism to bacterial acid resistance in general and specifically to acid resistance of *L. reuteri* is well understood (Rollan et al., 2003; Cotter and Hill, 2003; Foster, 2004; Su et al., 2011; Feehily and Karatzas, 2013). However, while arginine metabolism is known to increase acid resistance by intracellular consumption of protons and the production of ATP (Konings, 2002), mechanisms of glutamine-glutamate system mediated acid resistance remain to be elucidated. The γ-

carboxyl group of glutamine and γ-aminobutyrate (GABA) has a pK_A value of 4.25, therefore, the proton balance of decarboxylation and transport is dependent on the intra- and extra-cellular pH (Feehily and Karatzas, 2013). A contribution of glutamine deamidation to acid resistance was recently demonstrated in *E. coli* (Brown et al., 2008; Lu et al., 2013) but has not been demonstrated experimentally in lactobacilli. Moreover, genetic determinants of glutamine conversion in lactobacilli remain to be determined.

It was the aim of this study to determine whether glutamine conversion to glutamate improves the survival of *L. reuteri* in acidic conditions independent of its arginine conversion and glutamate decarboxylation. Moreover, the study aimed to determine whether arginine, glutamine, and glutamate-dependent systems for acid resistance are redundant, i.e providing a comparable level of protection at given conditions of acid stress, or complementary, i.e. optimally functional at different levels of acidity. Three putative glutaminase genes were identified in *L. reuteri* 100-23, a rodent isolate for which genome sequence data are available (Wesney and Tannock, 1979). The quantification of gene expression, and determination of the role of amino acids for survival and transmembrane potential of *L. reuteri* at acidic conditions were used to elucidate the contribution of arginine, glutamine, and glutamate to acid resistance in *L. reuteri*.

2. Materials and methods

2.1. Media, strains and growth conditions.

Lactobacillus reuteri 100-23 and 100-23Δ*gadB* (Su et al., 2011) were grown in modified MRS medium (mMRS, Gänzle et al., 1998) at 37°C. Ten mmol L^{-1} of glutamine were added where indicated. Frozen stock cultures of *L. reuteri* were inoculated on mMRS

agar plates, single colonies were inoculated in one mL mMRS medium, subcultured with 1% inoculum in mMRS medium, and grown to the exponential phase (5 h of incubation, OD_{600nm} = 0.5) or the late stationary phase (48 h of incubation, OD_{600nm} = 1.6).

2.2. Survival of L. reuteri in acid stress at pH 3.5 or pH 2.5.

To determine the relevance of different acid resistance mechanisms of *L. reuteri* at different pH values, the survival of *L. reuteri* 100-23 and 100-23Δ*gadB* was compared after acid stress in 100 mmol L^{-1} lactate buffer (pH 3.5) and in 100 mmol L^{-1} phosphate buffer (pH 2.5). Buffers were supplemented with 20 mmol L^{-1} arginine, glutamine, or glutamate as indicated, and the pH was re-adjusted with HCl to pH 3.5 or 2.5 after the addition of amino acids. Cells were grown to the early stationary phase of growth (14 – 18 h), harvested by centrifugation, and resuspended in lactate or phosphate buffers. Based on previous investigations of acid resistance of *L. reuteri* 100-23 (Su et al., 2011), cells were incubated at 37 ºC for 24 h in the pH 3.5 buffer, or at 37 ºC for 10 h in the pH 2.5 buffer prior to the enumeration of viable cell counts by plating. Because the buffering capacity exceeded the substrate concentration, the pH remained unchanged throughout incubation (data not shown). Results are expressed as mean value ± standard deviation of quadruplicate independent experiments. Statistical analysis was performed using Student's *t*-test.

2.3. Analysis of amino acids using high performance liquid chromatography (HPLC)

Samples of sourdoughs fermented with *L. reuteri* 100-23 were additionally analyzed with regards to viable cell counts, and the concentrations of amino acids. Amino acids were quantified by HPLC and post-column derivatization with *o*-phthaldialdehyde (OPA) as described (Sedgewick et al., 1991).

2.4. Measurement of intracellular pH (pH_{in})

The effect of amino acids on transmembrane proton potential was determined by quantifying internal pH with a conjugated fluorescent pH probe 5(6)-carboxyfluorescein diacetate succinimidyl ester (cFDASE; Sigma) essentially as described (Breeuwer et al. 1996). Modifications of the method were required to maintain *L. reuteri* metabolically active and with a measurable transmembrane potential throughout the staining protocol (Gänzle and Vogel, 2003). In brief, cells were harvested from overnight cultures in mMRS, harvested by centrifugation, and washed and resuspended in 50 mmol/L citrate phosphate buffer (pH 5.0). Subsequently, the cells were incubated in the presence of 10 μmol/L cFDASE at 37 °C for 15 min. After labeling with cFDASE, cells were collected by centrifugation and resuspended to an OD (600 nm) of 10.0 in 50 mmol/L citrate phosphate buffer (pH 5.0) containing 10 mmol/L of maltose and fructose, 0.4 mmol/L $MgSO_4$, and 0.3 mmol/L $MnSO_4$ to eliminate non-conjugated probe. The cells were subsequently diluted to an OD (600 nm) 1.0 in 20 mmol/L citrate phosphate buffer (pH 4.0), 100 mmol/L lactate buffer (pH 4.0), or 100 mmol/L acetate buffer (pH 5.0) supplemented with 20 mmol/L arginine, glutamine, or glutamate as indicated. The cultures and all buffers were maintained at 37°C to maintain the cells metabolically active. The temperature of centrifuges was set to room temperature and the duration of centrifugation steps was limited to 3 min.

The internal and external pH values were determined at different time points after incubation at 37°C. Fluorescence intensities were measured using a microtiter plate reader (Varioskan Flash; Thermo Fisher Scientific; Nepan, ON) at the excitation wavelength of 490 nm and 440 nm by rapidly altering the monochromator between both

wavelengths. The emission wavelength was 525 nm, and the excitation and emission slit width was 5 nm. Calibration curves for *L. reuteri* and its mutant *L. reuteri* 100-23Δ*gadB* were determined in 50 mmol/L citrate phosphate buffers with pH values ranging from 4 to 8. The pH_{in} and pH_{out} were equilibrated by addition of nigericin (Sigma) and valinomycin (Sigma) to a final concentration of 5 μmol/L, and the ratios were determined as described previously (Gänzle and Vogel, 2003). Calibration curves were established for each batch of labeled cells. Data presented are means ± standard deviations from three independent experiments. Statistical analysis was performed using Student's *t*-test.

2.5. Monitoring of the transmembrane electrical potential (ΔΨ)

Changes in transmembrane potassium potential in *L. reuteri* 100-23 and its mutant *L. reuteri* 100-23Δ*gadB* were monitored using the fluorescent probe 3,3'-dipropylthiacarbocyanine [$DiSC_3(5)$; Invitrogen] (Gänzle and Vogel, 2003). The harvested cells were washed once with 20 mmol/L citrate phosphate buffer (pH 6.5), 20 mmol/L citrate phosphate buffer (pH 4.0), or 100 mmol/L lactate buffer (pH 4.0), containing 10 mmol/L of maltose and fructose as carbon source and 0.4 mmol/L $MgSO_4$ and 0.3 mmol/L $MnSO_4$. After centrifugation, the cells were resuspended to an OD (600 nm) of 0.5 in the same buffers supplemented with 20 mmol/L of arginine, glutamine, or glutamate as indicated. Measurements were carried out at time points of 0, 12, and 24 h after incubation at 37°C. The cells were transferred to a microtiter plate containing $DiSC_3(5)$ to a final concentration of 5 μmol/L. The cells were incubated for about 10 minutes to equilibrate the internal and external dye concentrations, followed by the addition of the proton ionophore nigericin (1 μmol/L) and the potassium ionophore valinomycin (1 μmol/L). Fluorescence measurements were performed using a microtiter

plate reader (Varioskan Flash) with excitation and emission wavelengths of 643 and 666 nm, respectively (slit widths of 5 nm). The data shown are representative of at least three independent experiments with consistent results.

2.6. RNA isolation and cDNA library construction from L. reuteri strains in mMRS, mMRS-Gln, acidified mMRS, and sourdough

To quantify the expression of *gls1, gls2, gls3, adi,* and *gadB* of *L. reuteri* during growth, mMRS medium or mMRS medium containing 10 mmol L^{-1} glutamine was used to inoculate *L. reuteri* 100-23 and 100-23Δ*gadB*. Samples were taken from cultures grown to the exponential phase (5 h incubation, corresponding to an OD_{600nm} of 0.3 – 0.4) or to the stationary phase (48 h). Two volumes of RNAprotect Bacteria Reagent (Qiagen, Mississauga, ON, Canada) were added to 1 mL of culture to maintain RNA integrity. Cells were harvested by centrifugation and stored at -80 ºC.

To determine the effect of acid stress on *gls1, gls2, gls3, adi*, and *gadB* expression in *L. reuteri* 100-23, the strain was grown to the exponential phase. Cells were centrifuged, and resuspended in mMRS acidified to pH 3.5 with HCl. *L. reuteri* acidifies mMRS to a pH of 3.5; i.e. this pH represents the growth / no growth interface. After 40 min of incubation in mMRS (pH 3.5) at 37°C, cultures were mixed with RNAprotect Bacteria Reagent and cell pellets were harvested and stored at -80 ºC.

Sourdoughs were prepared with 1 mL of an overnight culture of *L. reuteri* 100-23, 10 mL sterile tap water, and 10 g whole wheat flour. Dough was incubated at 37 ºC and samples were taken after 5 h (exponentially growing cells, corresponding to a pH of about 4.5) or 48 h (stationary phase of growth, corresponding to a pH of about 3.6). Aliquots of 0.5 g sourdough were mixed with 3 mL of RNAprotect Bacteria Reagent (Qiagen), incubated

at room temperature for 10 min, and the solids were removed by centrifugation at 400 *g* for 10 min. Cells in the supernatant were harvested by centrifugation and the cell pellets were stored at -80ºC prior to RNA isolation.

RNA was isolated from cell pellets using Trizol reagent according to the manufacturer's instructions (Molecular Research Center, Inc, Cincinnati, the USA). Contaminant genomic DNA was digested by DNase treatment, and cDNA libraries were generated by reverse transcription as described (Schwab and Gänzle, 2006).

2.7. Relative quantification of gene expression by quantitative PCR (qPCR)

Specific primers targeting *gls1, gls2, gls3, adi*, and *gadB* (Table 1) were used for qPCR amplification, which was performed using the QuantiFast SYBR green master mixture (Qiagen) in a 7500 Fast Real Time-PCR System (Applied Biosystems, USA). Primers were designed based on the genome sequence of *L. reuteri* 100-23 (GenBank Accession number AAPZ02000002.1). To verify the specificity of the primers, qPCR reactions were carried out with chromosomal DNA as template for subsequent determination of the size and melting point of the amplicons. Chromosomal DNA isolated from *L. reuteri* 100-23 and DNase-treated RNA samples were used as positive and negative controls, respectively, in all RT-qPCR reactions. Normalized gene expression ratios were calculated according to Pfaffl (2001) using the gene *pho* coding for phosphoketolase in *L. reuteri* as reference gene, and exponentially growing cultures of *L. reuteri* 100-23 in mMRS as reference condition (N_0). The efficiencies of the PCR reactions were determined in PCR reactions with serial 10-fold dilutions of chromosomal DNA of *L. reuteri* 100-23 as template. The efficiencies (*E*) of PCR reactions targeting *pho, gls1, gls2, gls3, adi*, and *gadB* were 1.90, 1.90, 1.91, 1.89, 2.00, and 2.00, respectively. Results

196 are reported as means ± standard deviation of duplicate independent experiments
197 analysed in duplicate.

198 **3. Results**

199 *3.1. Identification of glutaminases in the genome of L. reuteri 100-23*

200 To identify the genetic determinants of glutamine deamidation in *L. reuteri* 100-23,
201 putative glutaminases were identified in the genome of *L. reuteri* 100-23 (Figure 2A).
202 The putative glutaminase gene *gls3* is located adjacent to the glutamate decarboxylase
203 *gadB* (Su et al., 2011) and the putative glutamate/GABA antiporters *gadC1* and *gadC2*.
204 The genes *gls1* and *gls2* are not located in the vicinity of other genes involved in
205 glutamine metabolism or transport (Figure 2A). The protein sequences of Gls1, Gls2, and
206 Gls3 in *L. reuteri* 100-23 are 44% to 66% identical to each other and 28% to 38%
207 identical to glutaminases of *E. coli* W3110 (YbaS and YneH) and *B. subtilis* ATCC
208 23857D-5 (YbgJ and YlaM) (Brown et al., 2008, Figure 2B). Serine-dependent β-
209 lactamases, penicillin-binding proteins, and glutaminases are assigned into a large protein
210 family; alignment of the glutaminase protein sequences confirmed that the conserved
211 catalytic residues S-X–X-K (S60-K63 in YbaS, β-lactamase motif I), the β-lactamase
212 motif III (K259-S-G261 in YbaS), and the incomplete β-lactamase motif II (S160 in
213 YbaS) were also conserved in the three glutaminases of *L. reuteri* (Brown et al., 2008,
214 Figure 2C, and data not shown). Moreover, of the 40 amino acid residues that are
215 conserved in 8 eukaryotic and prokaryotic glutaminases (Brown et al., 2008), 37, 30, and
216 36 residues were also conserved in *gls1*, *gls2*, and *gls3*, respectively. PCR analysis of
217 cDNA libraries demonstrated that *gls1*, *gls2*, and *gls3* are expressed (Table 2 and data not
218 shown). Protein homology, the presence of conserved catalytic domains, and gene

219 expression indicate that *gls1*, *gls2*, and *gls3* of *L. reuteri* 100-23 code for three functional
220 glutaminases.

221 *3.2. Survival of L. reuteri in acid stress at pH 3.5 and pH 2.5*

222 The survival of *L. reuteri* in acidic conditions was determined in lactate buffer (pH 3.5)
223 to mimic conditions in the forestomach of rodents and in sourdoughs (Gänzle et al.,
224 1998), and in phosphate buffer (pH 2.5) to imitate gastric transit (Ward and Coates,
225 1987). Lactate and phosphate buffers were supplemented with amino acids to evaluate
226 their contribution to survival (Figure 3). Previous experimentation in phosphate buffer,
227 pH 2.5 confirmed amino acid conversion under conditions of lethal acid stress (Su et al.,
228 2011). Glutamine and glutamate but not arginine improved survival at pH 2.5 (Figure
229 3A). To determine whether the protective effect of glutamine on the survival of *L. reuteri*
230 100-23 in acidic condition at pH 2.5 is mediated by glutamine deamidation or by
231 glutamate decarboxylation (Figure 1), the experiment was also carried out using *L.*
232 *reuteri* 100-23Δ*gadB*. Disruption of *gadB* eliminated the protective effect of glutamate on
233 the survival of *L. reuteri* 100-23Δ*gadB*. However, glutamine remained protective for *L.*
234 *reuteri* 100-23Δ*gadB*, demonstrating that glutamine deamidation protects against acid
235 stress in the absence of glutamate decarboxylation (Figure 3B). Arginine provided no
236 protection at pH of 2.5, however, survival at pH 3.5 was improved by arginine addition
237 (Figure 3C). In contrast, glutamine or glutamate had no significant protective effect at pH
238 3.5, indicating that the effect of amino acid conversions on acid resistance is dependent
239 on the ambient pH.

240 *3.3. Effect of amino acid metabolism on the transmembrane potential (ΔpH and ΔΨ).*

241 The divergent effect of arginine and glutamate or glutamine on survival at acidic
242 conditions implies that arginine- and glutamate-mediated acid resistance is dependent on
243 different mechanisms. The role of amino acid-based acid resistance in *L. reuteri* 100-23
244 strain was evaluated by measuring changes in the intracellular pH and the dissipation of
245 the transmembrane potassium potential of *L. reuteri.* To determine if the protective effect
246 of glutamine and glutamate on the survival of *L. reuteri* 100-23 under acidic condition is
247 mediated by glutamine deamidation or by glutamate decarboxylation, the experiments
248 were also carried out with *L. reuteri* 100-23Δ*gadB*.
249 The intracellular pH was measured by the cFDASE method. This fluorescent dye has a
250 pK_A value of 6.5, which is suitable for the determination of pH_{in} values in the range of
251 5.0 – 7.5. Experiments performed at conditions imposing lethal acid stress on *L. reuteri*
252 (pH 2.5 or pH 3.5) indicated that pH_{in} decreased rapidly to values below 4.0, i.e. to values
253 outside of the pH range that can be determined with cFDASE (data not shown).
254 Therefore, the pH_{in} was additionally determined at conditions imposing sublethal acid
255 stress at pH 4.0 (citrate phosphate and lactate buffers) and 5.0 (acetate buffer).
256 The pH_{in} of *L. reuteri* 100-23 decreased dependent on the pH_{ex} (Figure 4). Results
257 obtained in presence of arginine confirmed that the arginine deiminase (ADI) pathway
258 consumes intracellular protons, and causes the alkalization of the fermentation substrate
259 (Konings, 2002, Figure 1 of the online supplemental material). The intracellular pH
260 increased in all of the three buffers when arginine was added (Figure 4). Arginine
261 conversion caused a higher increase of the pH_{in} in *L. reuteri* 100-23Δ*gadB*. Glutamate
262 conversion significantly increased the pH_{in} in citrate-phosphate buffer, pH 4.0; this effect
263 was not observed with glutamine or in other buffer systems. *L. reuteri* 100-23 and

100-23Δ*gadB* remained viable (data not shown) during incubation at pH 4.0 despite the apparent lack of a transmembrane proton potential (Figure 4 and data not shown).

The contribution of the transmembrane potassium potential (ΔΨ) to amino acid-based acid resistance was investigated with the cationic fluorescent probe $DiSC_3(5)$. During measurements in the presence or absence of amino acids, the membrane potential was manipulated with the proton-ionophore nigericin and the potassium ionophore valinomycin. In citrate phosphate buffer (pH 6.5), nigericin addition increased the membrane potassium potential because the loss of the ΔpH is compensated by an increase in the ΔΨ, as evidenced by decreased fluorescence intensity (Figure 5). Valinomycin addition abolished the potassium potential, resulting in an increase in fluorescence intensity (Figure 5). Monitoring the changes of membrane potential in the presence of amino acids under this condition indicated that the ΔpH and the ΔΨ were higher in *L. reuteri* 100-23 and 100-23Δ*gadB* incubated with arginine when compared to the control.

To further confirm the involvement of ΔΨ in the amino acid-based acid resistance, the membrane potential was monitored in cells incubated at a pH_{ex} 4.0. Proton-ionophores specifically increase the permeability of the membrane to protons. However, at a pH_{ex} 4.0, the proton-ionophore nigericin aggregates in trimers that also transfer potassium ions across the hydrophobic membrane (Toro et al., 1987). At an external pH of 4.0, the addition of nigericin thus slowly increased $DiSC_3(5)$ fluorescence, contrasting the decrease observed at a high pH. Because *L. reuteri* lacks a transmembrane ΔpH under this condition, this reflects a slow dissipation of the transmembrane potassium gradient (Figure 5). Valinomycin addition resulted in a rapid dissipation of the transmembrane potassium gradient, and a rapid increase in $DiSC_3(5)$ fluorescence in all cultures. At a

287 pH_{ex} of 4.0, the addition of valinomycin showed a higher increase of probe fluorescence
288 when glutamate was present, indicating a higher in transmembrane potassium potential in
289 presence of glutamate. This increased probe fluorescence in presence of glutamate was
290 not observed with *L. reuteri* 100-23Δ*gadB*, demonstrating that the effect is attributable to
291 glutamate decarboxylation. Taken together, arginine conversion had the most pronounced
292 effect on the intracellular pH while glutamate decarboxylation and electrogenic exchange
293 Glu and GABA additionally increased the ΔΨ when the intracellular pH was less than 4.2.

294 *3.4. Expression of glutaminases, glutamate decarboxylase, and arginine deiminase in*
295 *L. reuteri 100-23*

296 The expression of amino acid-based mechanisms of acid resistance was quantified in
297 stationary phase cells growing in mMRS, in exponentially growing cells after exposure to
298 acid stress, and in mMRS supplemented with glutamine (Table 2). Acid stress induced by
299 the stationary phase of growth, or by exposing exponentially growing cells to pH 3.5,
300 consistently resulted in over-expression of *gls3* and *gadB* (Table 2). Supplementation of
301 mMRS with 10 mmol L^{-1} glutamine resulted only in a two-fold increase of *gls3*
302 expression after 48 h of incubation. Expression levels of *gls1* and *gls2* were not
303 influenced by culture conditions in mMRS (Table 2). Expression levels of *adi* were
304 essentially unchanged after acid stress, and reduced in stationary phase cells.

305 The amino acid availability and the organic acid production in cereal substrates differ
306 substantially from laboratory media; gene expression was therefore also quantified in
307 wheat sourdough fermentations with *L. reuteri* 100-23 (Table 2). Analysis of amino acid
308 concentrations in sourdough confirmed that arginine was converted to ornithine and
309 glutamine was converted to γ-aminobutyrate (Stromeck et al., 2011, and data not shown).

After 48 h of fermentation, ornithine and GABA accumulated to 1.43 ± 0.12 and 2.66 ± 0.12 mmol kg^{-1}, respectively. Stationary phase cultures in wheat sourdough over-expressed *gls3* and *gadB* more than 100-fold; additionally, *gls2* was over-expressed (Table 2). *Adi* expression was reduced in exponentially growing cells in sourdough.

3.5. Expression of glutaminases, glutamate decarboxylase, and arginine deiminase in L. reuteri 100-23ΔgadB

To determine whether disruption of one acid resistance mechanism is compensated by over-expression of other acid resistance genes, gene expression levels in *L. reuteri* 100-23Δ*gadB* were quantified relative to the wild type strain *L. reuteri* 100-23 growing cells in mMRS medium (N_0). Deletion of *gadB* resulted in over-expression of *adi* (4.5 ± 1.2) and *gls3* (28.4 ± 10.4) at 48 h of cultivation.

4. Discussion

The present study demonstrated that glutamine deamidation improves survival rate of *L. reuteri* in pH 2.5 conditions regardless of glutamate decarboxylation. Bioinformatic analyses of glutaminase genes in *L. reuteri* 100-23, and the relative quantification of their gene expression indicate that this strain harbors three glutaminase genes. Gene expression data suggest that glutamine conversion in *L. reuteri* 100-23 is predominantly attributable to the *gls3-gadB* operon.

Glutamate, or arginine conversion consumes intracellular protons and thus increase acid resistance (Konings, 2002; Cotter and Hill, 2003; Feehily and Karatzas, 2013; Lu et al., 2013). Arginine is decarboxylated to agmatine by *Escherichia coli* and related organisms (Foster, 2004; Richard and Foster, 2004); lactic acid bacteria convert arginine to ornithine

via the arginine deiminase (ADI) pathway (Konings, 2002). Glutamine was recently shown to improve acid resistance of *E. coli* (Lu et al., 2013). The amino acid composition of cereal proteins (Wieser, 2007), and the organization of genes coding for glutamine and glutamate metabolism imply a role of glutamine in acid resistance in *L. reuteri* 100-23 (Vermeulen et al., 2007; Su et al., 2011). This study demonstrates that the acid resistance of *L. reuteri* that is achieved by glutamine deamidation is comparable to that achieved by glutamate decarboxylation. *L. reuteri* 100-23 thus maintains three mechanisms for amino acid-mediated acid resistance; urease activity and fructan formation additionally improve acid resistance (Figure 1 and 6). Moreover, *L. reuteri* 100-23 harbours three different glutaminases. The coordinated over-expression of *gls3* and *gadB*, both located on the same operon, implies that these genes are mainly responsible for glutamine and glutamate conversion in *L. reuteri* 100-23. However, disruption of glutamate decarboxylase in *L. reuteri* 100-23Δ*gadB* was compensated by over-expression of *adi* and *gls3*. This result conforms to the improved pH homeostasis of *L. reuteri* 100-23Δ*gadB* in presence of arginine as well as the conditional over-expression of the ADI pathway in *Lactococcus lactis* in absence of glutamate (Mazzoli et al., 2010).

Mechanisms of acid resistance in *L. reuteri* employ the substrates urea, glutamate / glutamine, and arginine. Urea is available in mammalian gastric environments but absent in food fermentations; arginine and glutamine are released by proteolysis from cereal proteins (Thiele et al., 2002). Moreover, the contribution of amino acid conversions to pH homeostasis is dependent on both the intracellular and the extracellular pH (Konings, 2002; Tsai et al., 2013; Ma et al., 2013). The majority of studies related to glutamate- or arginine-mediated bacterial acid resistance simulated gastric survival at pH 2 – 2.5

355 (Cotter and Hill, 2003; Foster, 20045; Feehily and Karatzas, 2013). At pH 2, glutamate
356 decarboxylation but not arginine decarboxylation improved survival of *E. coli* (Hersh et
357 al., 1996). This study compared the survival of *L. reuteri* at pH 2.5 in a phosphate
358 buffered system with the survival at pH 3.5 in a lactate-buffered system to match
359 conditions of the gastric environment at pH 2.5, or the rodent forestomach and long-time
360 sourdough fermentations (Ward and Coates, 1987; Gänzle et al., 1998). Arginine
361 protected *L. reuteri* at pH 3.5 while glutamine or glutamate protected *L. reuteri* at pH 2.5.
362 These findings demonstrate that acid resistance mechanisms are complementary with
363 respect to their functions under different conditions.

364 *L. reuteri* did not maintain a high intracellular pH at acidic pH_{ex}. Likewise, beer spoiling
365 *L. brevis* survives at low pH values without maintaining a large transmembrane ΔpH
366 (Schurr et al., 2013). The transmembrane ΔpH was lower at high external lactate
367 concentrations, likely because pH homeostasis by H^+/lactate symport is less effective at
368 high external lactate concentrations (Konings and Otto, 1983). The ability of lactobacilli
369 to survive with a low pH_{in} contrasts observations in *E. coli*. In *E. coli,* acidification of the
370 cytoplasm below pH 5.0 compromises survival (Foster, 2004, Ma et al., 2013).
371 Arginine conversion by *L. reuteri* consumes two intracellular protons; the production of
372 ATP enables the additional extrusion of protons by F_0F_1-ATPase (Konings, 2002; De
373 Rollan et al., 2003). This study confirmed cytoplasmic alkalization as a result of arginine
374 conversion in *L. reuteri*. Arginine conversion thus provides protection predominantly at
375 moderate levels of acidity.

376 The effect of glutamate conversion on the pmf depends on the extra- and intracellular pH.
377 The consumption of protons and the polarization of the membrane differ depending on

the species of glutamate (Glu^{-1}, Glu^{-0}, or Glu^{-1}) and GABA ($GABA^{0}$ or $GABA^{+1}$) which are transported. At pH values of less than 4.0, Glu^{0} is the predominant species because the γ-carboxyl group (pK_A 4.25) is mostly protonated. Decarboxylation of glutamate eliminates one cytoplasmic proton; $Glu^{0}/GABA^{+1}$ antiport additionally exports one charge (Figure 6). We observed that glutamate decarboxylation increased the cytoplasmic pH and additionally polarized the membrane in *L. reuteri*, suggesting comparable mechanisms as previously described in *E. coli* (Ma et al., 2013).

Glutamate or glutamine conversion improved survival of *L. reuteri* only at pH 4.0 or less. Under these conditions, the *gls3-gadB* operon is highly expressed and Glu^{0} and $GABA^{+1}$ are the predominant species. However, the increase of the cytoplasmic pH was observed only in citrate phosphate buffer and it was small when compared to arginine. This suggests that membrane polarization by glutamate decarboxylation may be more relevant for survival of *L. reuteri*. The deamidation of glutamine is also predicted to consume one proton (Figure 6) but this study failed to detect cytoplasmic alkalinisation. In *E. coli*, GadC also transports glutamine (Lu et al., 2013) but the specific role of *gadC1* and *gadC2* in *L. reuteri* remains unknown.

Genetic and physiological analyses of *L. reuteri* show that different lineages of *L. reuteri* have evolved with their hosts (Frese et al., 2011). Strains from different host-adapted lineages particularly differ with respect to mechanisms of acid resistance (Frese et al., 2011). Rodent isolates but not human isolates of *L. reuteri* exhibit urease activity and the *gls3-gadB* operon is found only in rodent isolates (Frese et al., 2011). The substrates urea and glutamine are present in gastric environments; urease as well as *gls3* and *gadB* are over-expressed by *L. reuteri* 100-23 during gastric transit (Wilson et al., 2011).

Conversely, histidine decarboxylase is present only in human-lineage strains (Saulnier et al., 2011). Genetic differences between rodent- and human-adapted strains of *L. reuteri* may reflect that *L. reuteri* colonizing the rodent forestomach are exposed to gastric acidity during subsequent passage of the digesta. In contract, human-lineage *L. reuteri* colonize the colon and are thus not exposed to gastric acidity.

Multiple amino acid-based acid resistance systems are also relevant for the prevalence of *L. reuteri* in the Type II sourdoughs (Vogel et al., 1999; Su et al., 2011). Glutamate decarboxylation increased the competitiveness of *L. reuteri* 100-23 in sourdough (Su et al., 2011). This study provides evidence that arginine is more effective than glutamine / glutamate conversions under conditions prevailing during long-term sourdough fermentations. It is noteworthy that metabolic pathways in *L. reuteri* that contribute to acid resistance (Figure 1) also alter the competitiveness in sourdough fermentations, and improve flavour and taste of bread (Su et al., 2011; Thiele et al., 2002; Vermeulen et al., 2007). The analysis of genetic determinants of glutamine conversion in lactobacilli thus facilitates the selection of starter cultures for improved competitiveness in long–term fermentations, or improved bread quality.

5. Acknowledgements

Albert Innovates Biosolutions, ACIDF, the Alberta Barley Commission, and NSERC are acknowledged for funding. Alma Fernanda Sanchez-Maldonado acknowledges support from CONACYT; Michael Gänzle acknowledges the Canada Research Chairs Program for funding.

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531

Figure legends

Figure 1. Metabolic pathways in *L. reuteri* contributing to acid resistance.

Panel A. Strain-specific glutamate decarboxylase (GadB) improves survival at pH 2.5 (Syu et al., 2011). Preliminary evidence indicates that glutamine deamidation contributes to acid resistance independent of glutamate decarboxylation (Su et al., 2011; Vermeulen et al., 2007). The decarboxylation of glutamate and the consumption of a proton increase the alkalinity of the cytoplasm at pH higher then 4.25 (Feehily and Karatzas, 2013). **Panel B.** The conversion of arginine to ornithine contributes to acid resistance in all strains of *L. reuteri* (Rollan et al., 2003; De Angelis and Gobbetti, 2004). **Panel C.** Histidine decarboxylation contributes to acid resistance of lactobacilli and was described for a human isolate of *L. reuteri* but is absent in rodent isolates (Konings et al., 2002; Freese et al., 2011; Stromeck et al., 2011; Thomas et al., 2012). **Panel D.** Urease activity contributes to acid resistance in some rodent isolates of *L. reuteri* (Wilson et al., 2011). **Panel E**. The strain-specific formation of exopolysaccharides (reuteran, levan, or inulin) and oligosaccharides by glucansucrases (GtfA) or fructansucrases (FtfA) protects against membrane-active inhibitors and improve stationary phase survival (Schwab and Gänzle, 2006; Kaditzky et al., 2008). GadC1 and GadC2, glutamate / GABA or glutamine / GABA antiporters; ArcD, arginine-ornithine antiporter; HcdA and HcdP, histidine decarboxylase and histidine-histamine antiporter. Ion charge assigned to molecules reflect intracellular and extracellular pH-values higher than 4.25.

Figure 2. Genes coding for glutaminases in *L. reuteri* 100-23. **Panel A.** Representation of genetic loci coding for glutaminases in *L. reuteri* 100-23. Nucleotide sequences and annotations were retrieved from GenBank with accession numbers

AAPZ02000001.1:333174..334094, AAPZ02000001.1:1455778..1456692, and AAPZ02000002.1:548219..549139 for genes *gls1*, *gls2*, and *gls3*, respectively. **Panel B**. Protein identity (%) of glutaminases in *L. reuteri* to the glutaminases YbgJ and YlaM in *B. subtilis*, and YbaS and YneH in *E. coli*. **Panel C**. Sequence alignment for conserved domains of glutaminases in *L. reuteri* to glutaminases in *E. coli* (YbaS and YneH) and *B. subtilis* (YbgJ and YlaM) (Brown et al., 2008). Numbers correspond to the YbaS sequence; highly conserved amino acids (Brown et al., 2008) are marked by asterisks and deviating amino acids in Gls1, Gls2, or Gls3 are underlined.

Figure 3. Survival of *L. reuteri* 100-23 **(Panels A and C)** and *L. reuteri* 100-23Δ*gadB* **(Panel B)** in acid stress. Cells were incubated in phosphate buffer at pH 2.5 for 10 h **(Panels A and B)** or in lactate buffer at pH 3.5 for 24 h **(Panel C)**. Control treatments were carried out in phosphate or lactate buffers (cont.); arginine, glutamine, or glutamate were added to a final concentration of 20 mmol L^{-1} as indicated. Data are shown as means ± standard deviation of quadruplicate independent experiments. Data obtained for the same strain at the same pH that do not share a common superscript are significantly different ($p<0.05$).

Figure 4. Effect of arginine, glutamine, and glutamate on the pH_{in} of *L. reuteri* 100-23 and its isogenic mutant *L. reuteri* 100-23Δ*gadB* after 0 h (black columns) and 24 h (grey columns) of incubation at 37°C in citrate phosphate (pH 4.0), lactate (pH 4.0), and acetate (pH 5.0) buffers. Ratio of cFSE in stained cells of *L. reuteri* 100-23 were measured at excitations of 490 nm and 440 nm, and emission wavelengths of 525 nm. The fluorescence ratio where pH_{in} and pH_{ex} were equilibrated by incubation with nigericin (1 μM) and valinomycin (1 μM) is indicated to the right of the figure. The results are means

± standard deviations of three independent experiments. Values that differ significantly from the controls that were incubated under the same conditions without addition of amino acids are indicated by an asterisk ($p < 0.04$).

Figure 5. Effect of arginine (—), glutamine (⋯), and glutamate (– –) on the membrane potential of *L. reuteri* 100-23 and its isogenic mutant *L. reuteri* 100-23Δ*gadB* after 12 hours of incubation at 37 °C. Fluorescent traces of $DiSC_3(5)$ in stained cells of *L. reuteri* 100-23 were determined with at excitation and emission wavelengths of 643 and 666 nm, respectively. Nigericin and valinomycin were added to a final concentration of 1 μmol/L at the time points indicated by the arrows to check the dissipation of the ΔpH and ΔΨ, respectively. Control , –⋯. RFU, relative fluorescence units. The results are representative for three independent experiments with consistent results.

Figure 6. Glutamine conversion and acid resistance in *L. reuteri*. Glutamine is the predominant amino acid that is released from cereal proteins during sourdough fermentation, or during digestion. The glutamate / GABA antiporter in *E. coli* transports glutamine as well as (uncharged) glutamate (Lu et al., 2013); glutamine antiport in *L. reuteri* may thus also be mediated by one of GadC1 or GadC. At intracellular pH values below the pK_A of the glutamate side chain (4.25), uncharged glutamate is the main ion species and glutamine deamidation consumes one intracellular proton (Lu et al., 2013). In *L. reuteri* 100-23, Gls3 is the most highly expressed glutaminase and likely responsible for most of the glutamine conversion. Glutamate is converted to GABA by glutamate decarboxylase (GadB) (Su et al., 2011) with concomitant consumption of one proton. GABA is exported as cation. The exchange of uncharged glutamine or glutamate with

positively charged GABA creates an electrogenic potential in addition to the proton gradient established by glutamine deamidation and glutamate decarboxylation.

603 **Table 1.** List of primers used in RT-qPCR

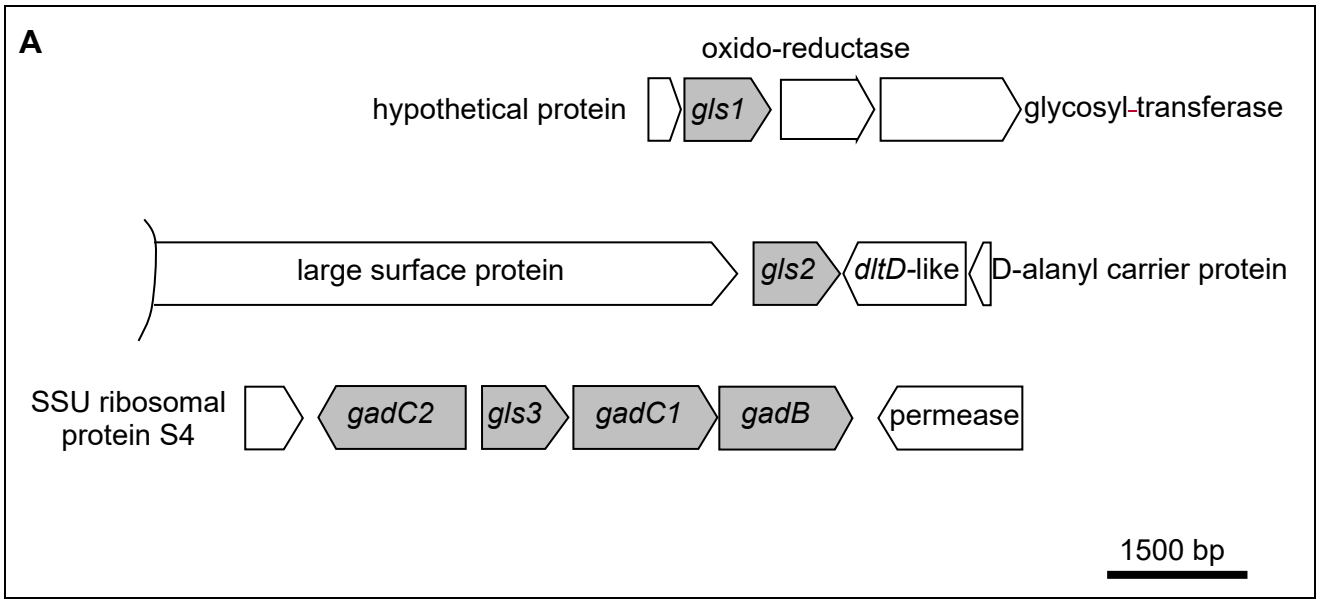
Target	Primer	Sequence (5'-3')
pho	*pho* Forward	GTA ACC TTC AAG GAA TCC
	pho Reverse	CGT CTT TAC GCA TTC CTT G
gls1	*gls1* Forward	AGC AGT TGA AGA ACA AGT CGG AA
	gls1 Reverse	CAT TGA GGG TGA TAG CGG GAT
gls2	*gls2* Forward	TAG GAG CAG TCT TGG CAA ATG AT
	gls2 Reverse	GAT CAA GAG CTG GAC TAA AAA TAC CA
gls3	*gls3* Forward	CAC ATT ATC CTC TCA ACC CAT TTA TC
	gls3 Reverse	ACC ATT GTT TGC TAA GAC TGC G
adi	*adi* Forward	CAG ACG CAC TGG CAG ATG AT
	adi Reverse	CCG ATA CAT GCC TGT TGG TCA C
gadB	*gadB* Forward	GAT GCT GCT TCT GGT GGA TTC T
	gadB Reverse	ATT CTC CTC CTA AGT AAC TAA CCT

604

Table 2. Relative quantification of the expression of the glutaminases *gls1, gls2,* and *gls3*, glutamate decarboxylase *gadB*, and arginine deiminase *adi* in *L. reuteri* 100-23. Gene expression was quantified in exponentially growing (5h) or stationary phase (48h) cultures in mMRS or mMRS-glutamine, after acid shock in mMRS, and during growth in sourdough. Gene expression in samples (N) was quantified relative to exponentially growing cells in mMRS medium (N_0); *pho* coding for phosphoketolase was used as reference gene. Results were calculated as N/N_0 and are reported as mean ± standard deviation of duplicate experiments analyzed in duplicate.

	mMRS	**mMRS**	**mMRS + 10 mmol L^{-1} Gln**		**sourdough**	
Gene	**48 h**	**acid shock[a)]**	**5 h**	**48 h**	**5 h**	**48 h**
gls1	0.4 ± 0.1	1.8 ± 0.2	1.7 ± 0.1	1.6 ± 0.6	0.3 ± 0.1	0.5 ± 0.2
gls2	0.9 ± 0.2	1.4 ± 0.3	0.8 ± 0.2	0.6 ± 0.3	0.2 ± 0.1	3.5 ± 1.5
gls3	4.3 ± 1.3	6.1 ± 0.8	2.0 ± 1.0	8.9 ± 0.6	3.7 ± 0.9	230 ± 110
gadB	7.6 ± 1.6	7.0 ± 1.0	n.d.	n.d.	0.9 ± 0.2	390 ± 76
adi	0.1 ± 0.0	0.5 ± 0.1	n.d.	n.d.	0.1 ± 0.0	0.8 ± 0.3

[a)] exponentially growing cells in mMRS (5 h) were resuspended in mMRS acidified to pH 3.5 and incubated for 40 min. n.d., not determined.



B.

Length (aa)	**YbgJ** 327	**YlaM** 309	**YbaS** 310	**YneH** 308	**Gls1** 306	**Gls2** 304	**Gls3** 306
Gls1	35%	38%	36%	33%	-	44%	66%
Gls2	32%	32%	28%	30%	/	-	45%
Gls3	36%	38%	36%	34%	/	/	-

C

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      24          56              117   257             276
YbaS  GQNADYIPFL  GDSDYRFALESISKV  NAGA  PGKSGVGGGILAVVP  IAAFSPPLDEDGNSVRG
YneH  GKVADYIPAL  GDAQERFSIQSISKV  NAGA  PAKSGVGGGIVAIVP  IAVWSPELDDAGNSLAG
YbgJ  GQSANYIPAL  GDWNVSFTMQSISKV  NAGA  PAKSGVSGGIMALVP  IGIYGPAIDEYGNSLTG
YlaM  GEVASYIPAL  GDVEKTFTLQSISKV  NAGA  PAKSGVSGGIMGISP  IGIFGPALDEKGNSIAG
Gls1  GQVATYIPAL  GASQVRFAIESVSKV  NSGA  PTKSGVGGGLVSAAP  IGIFSPALDHAGNSVAG
Gls2  GKVANYIPAL  GMAGTRFAIESIAKV  NSSA  PIKSGTGGGLLACAP  IGIFSPALDQHGNSLAG
Gls3  GKVANYIPAL  GNADVRFAIESISKV  NAGA  PTKSGVGGGLMSAAP  IGIFSPPLDNAGNSVAG
      *  * *** *  *     *   *  **  **** * ** * *      *       *     ***  *
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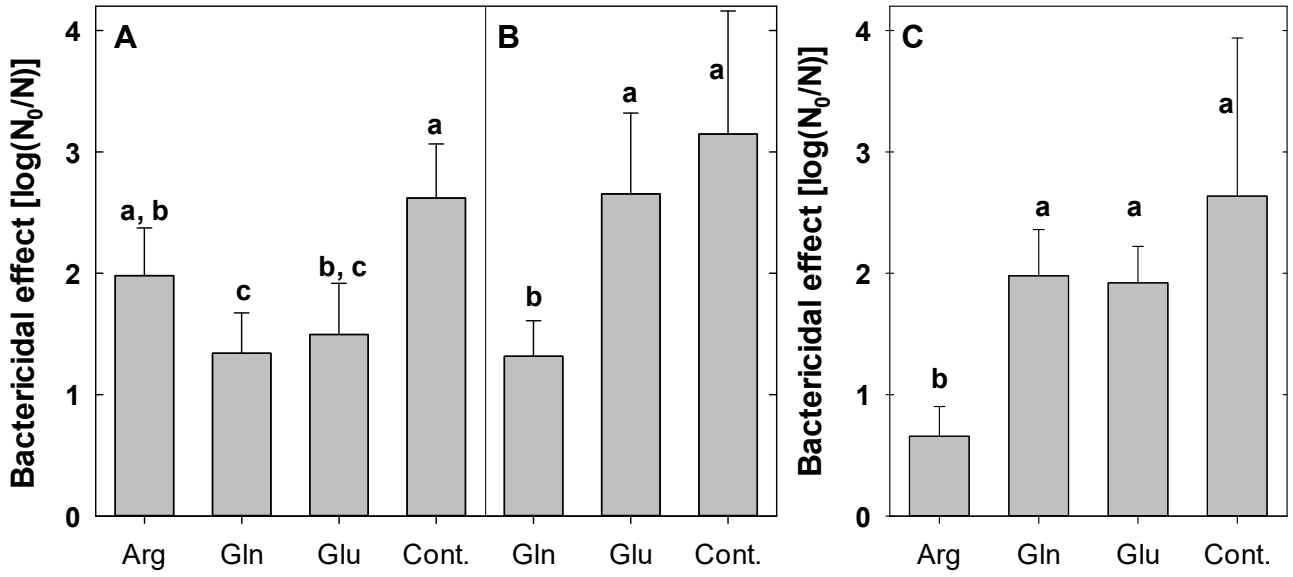
Figure 2 Teixeira et al.



620

621 **Figure 3** Teixeira et al.

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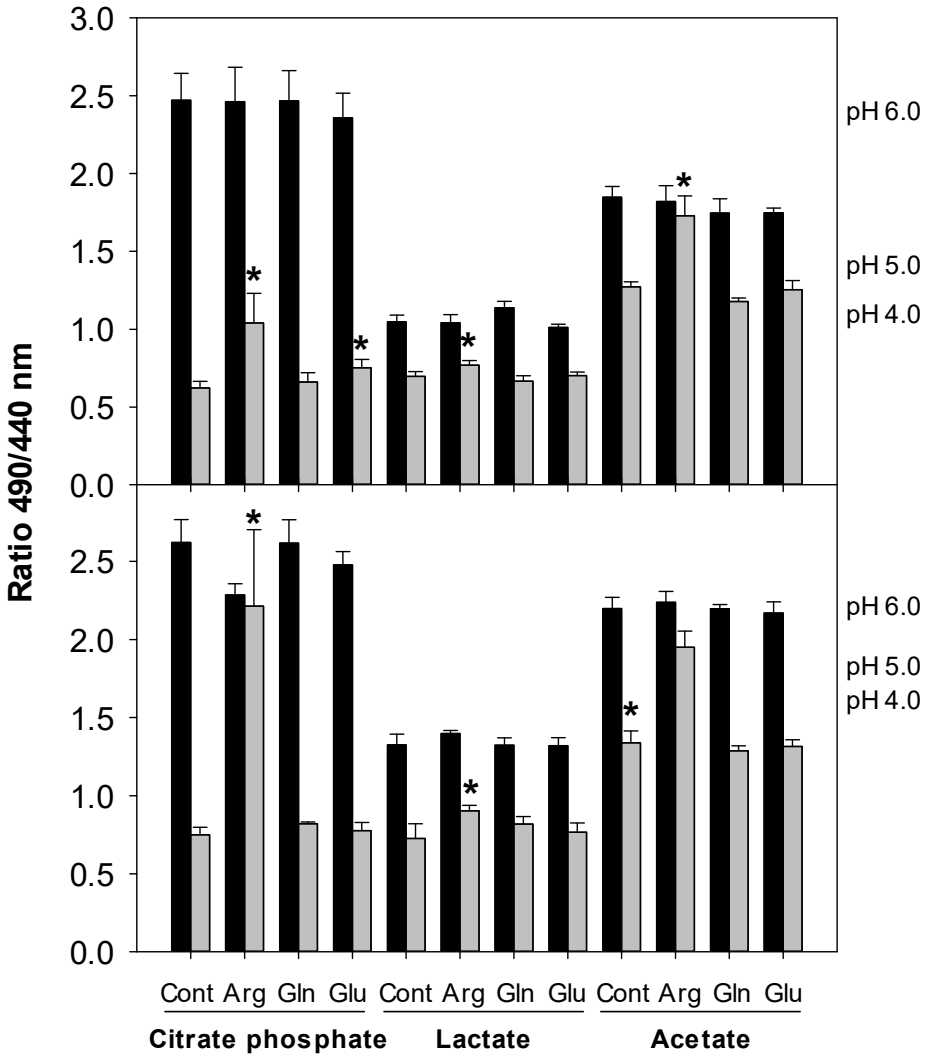

623 **Figure 4** Teixeira et al.

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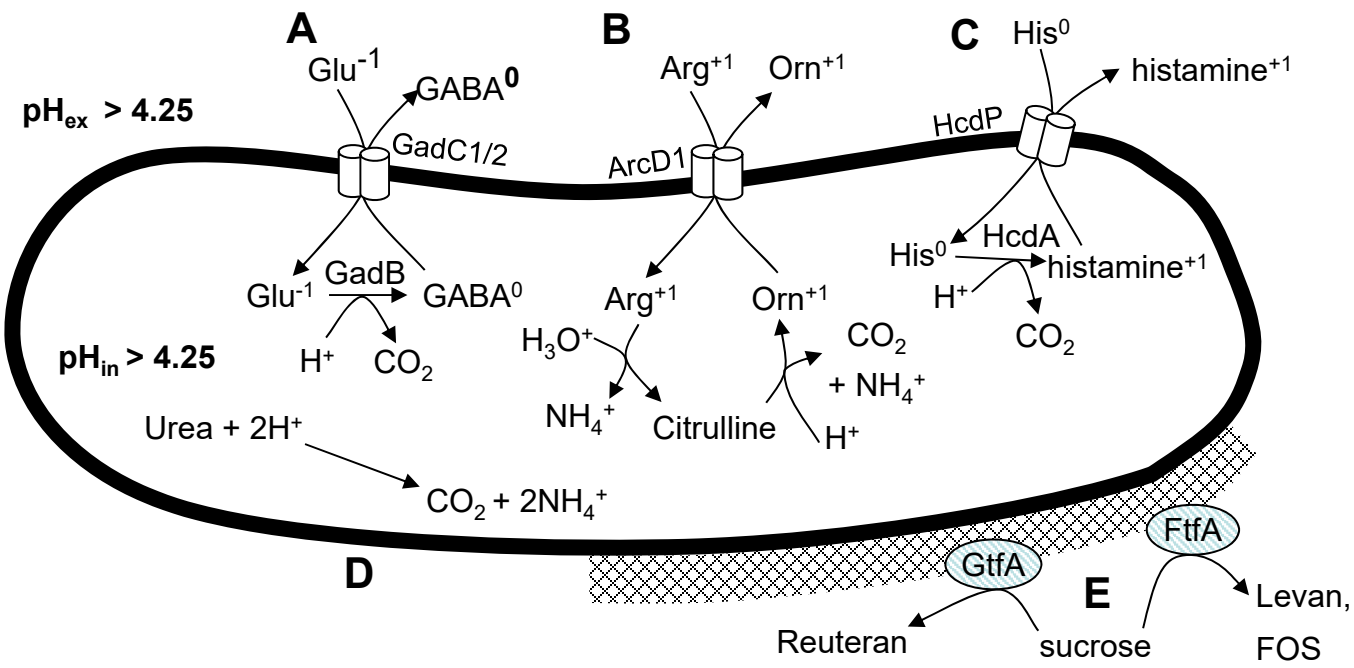

Figure 1. Teixeira et al.

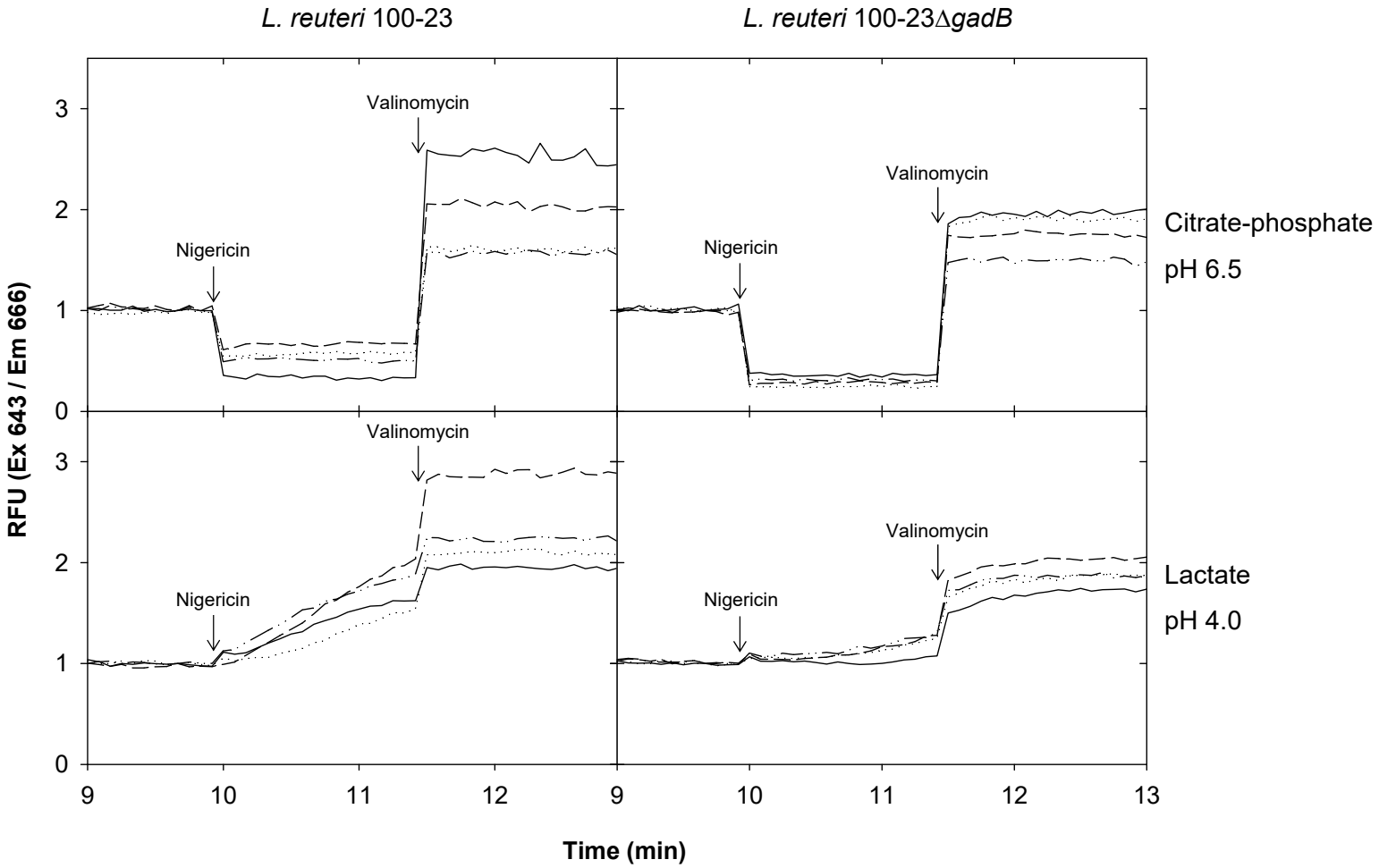

Figure 5. Teixeira et al.

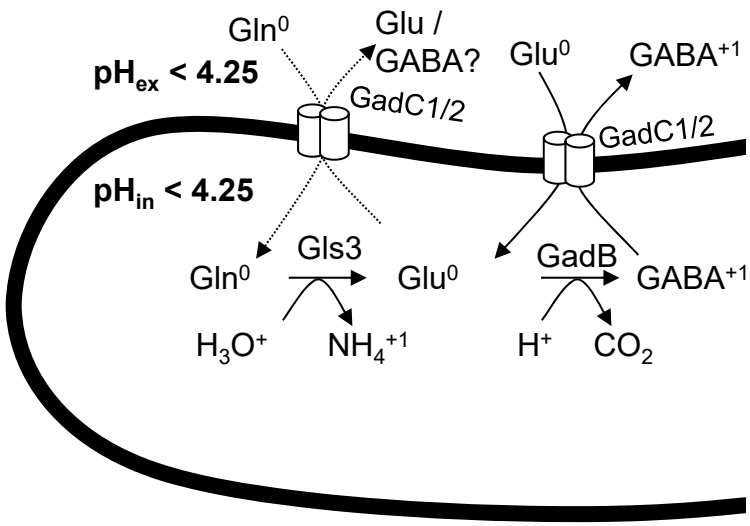

Figure 6. Teixeira et al.