Contribution of glutaminase activity in *Lactobacillus reuteri* to acid resistance and glutamine metabolism in sourdough

by

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Abstract

Sourdough is used as an additive in bread production for proper dough volume (leavening), or for desired dough acidity, or for dough texture and bread flavor improvement, or for bread shelf life extension. *Lactobacillus reuteri*, an intestinal isolate and a stable member of sourdough, prevails in Type II sourdough fermentation, owing to the elevated fermentation temperatures with long incubation time. The aim of this study was to understand the role of glutaminase activity in *L. reuteri* to acid resistance and glutamine metabolism throughout sourdough fermentation.

An isogenic deletion mutant *L. reuteri* 100-23 Δ*gls*1-2-3 was generated, by disruption of the three glutaminases (*gls*) in *L. reuteri* 100-23, for interpreting the role of glutaminase activity. Acid resistance mechanism was assessed at both pH 2.5 and 3.5 to mimic the gastric and sourdough environments; sourdough fermentation and sensory evaluation of sourdough bread were applied to establish the effect on glutamine metabolism.

Glutaminase-mediated deamidation contributes to the survival of *L. reuteri* 100-23 in both acidic environments (*in vitro*) and sourdough fermentations (*in vivo*) by converting glutamine to glutamate. Analysis of the glutamine / glutamate dependent acid resistance indicated that glutamine deamidation increases acid resistance independent of glutamate decarboxylation. Without the ability to convert glutamine, alternative acid tolerance mechanisms compensate (i.e. GAD; ADI; etc.) when *L. reuteri* can no longer rely on *gls*-mediated glutamine metabolism.

Evaluation of sourdough bread fermented with *L. reuteri* strains demonstrated a significant difference between the glutamate accumulating *L. reuteri* 100-23Δ*gad*B and the γ-aminobutyrate accumulating wild type *L. reuteri* 100-23. In contrast, bread produced with *L. reuteri* 100-23

Δ*gls*1-2-3, which does not convert glutamine to either glutamate or γ-aminobutyrate, was not different from bread produced with *L. reuteri* 100-23.

Glutamine conversion of sourdough lactobacilli contributes to acid resistance, and enhances the taste of bread. Extended information on acid resistance and glutamine metabolism of *L. reuteri* furthers the understanding of *L. reuteri* to cereal ecosystems, and results will contribute to the selection of strains that can be used as starter cultures for baking improvers (i.e. production of salt reduced bread).

Preface

(Research ethics approval)

Amendment/Renewal to Study has been Approved

Amendment/Renewal ID:	Pro00036093_REN2
Study ID:	MS2_Pro00036093
Study Title:	Sensory evaluation of bread
Study Investigator:	Michael Gaenzle
Description:	The amendment/renewal to the above study has been approved. Click on the link(s) above to navigate to the HERO workspace. Important: Please be reminded that the REMO system works best with Internet Explorer or Firefox.

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This thesis is an original work by Qianying Tao. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Sensory evaluation of bread”, No. Pro00036093_REN2, Feb 20, 2015.

No part of this thesis has been previously published.

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LAB: lactic acid bacteria

*gls*1: gene coding for glutaminase Gls1

*gls*2: gene coding for glutaminase Gls2

*gls*3: gene coding for glutaminase Gls3

*gad*B: gene coding for glutamate decarboxylase GadB

GLS: glutaminase

GAD: glutamate decarboxylase

Δ: mutant

Log: logarithmic

CFU: colony-forming unit

LB broth: Luria-Bertani broth

MRS broth: deMan-Rogosa- Sharpe broth

Erm: Erythromycin

BABA: β-aminobutyric acid

EA: ethanolamine

GABA: γ-aminobutyric acid

α –KG: α -ketoglutarate

PCR: polymerase chain reaction

HPLC: high-performance liquid chromatography

ANOVA: Analysis of variance

1 INTRODUCTION

1.1 Use of sourdough in bread production

Sourdough is a mixture of wheat and/or rye flour, water and/or salt, fermented by lactic acid bacteria and yeasts from flour (Gobbetti & Gänzle, 2013). In general, the dominant organisms in sourdoughs are lactic acid bacteria and elevated numbers of the co-existing yeasts, which are the key factors to the acidifying and leavening ability (Vogel et al., 1999). Owing to the cooperative activity of lactic acid bacteria and yeasts, as well as the interactive effects among bread making processes and ingredients, sourdough is a complex biological system (Gobbetti, 1998).

Sourdough is used as an additive in bread production for proper dough volume (leavening), or for desired dough acidity, or for dough texture and bread flavor improvement, or for bread shelf life extension (Su et al., 2011).

Sourdough fermentation profoundly affects the formation and liberation of flavor compounds in bread making process: wheat bread crumb made with sourdough showed higher content of alcohols and acids (Hansen & Hansen, 1996); bread produced with lactic acid bacteria sourdough was rated higher in terms of flavor intensity compared to conventional bread (Thiele et al., 2002). In regards to bread texture, exopolysaccharides formed by sourdough lactic acid bacteria have positive effect on the ability of dough water absorption and therefore improves bread volume and staling rate (Arendt et al., 2007). Moreover, the formation of organic acids during sourdough fermentation act as natural preservatives and are beneficial to bread shelf-life; a case in point is the inhibition of rope spoilage in wheat sourdough bread produced with *Lactobacillus plantarum*VTTE-78076, or *Lactobacillus brevis* (Katina et al., 2002).

Sourdough fermentation can be categorized into two types (namely, type I, and II) based on the technology used, which dictates the ecology of the dough. Type I sourdough is mainly used for leavening to achieve desired dough volume, maintained by continuous back slopping of active microbiota, in which short fermentation time and low fermentation temperature are preferred (Brandt, 2006). Type II sourdough is used in the food industry as baking improvers and typically fermented at 35 to 40°C for extended fermentation time up to 96 hours (Brandt, 2007).

Due to the particular fermentation conditions of type I and type II sourdoughs, the prevailing *Lactobacillus* strains differs. Lactic acid bacteria with various phenotype and genotype that dominate the sourdough fermentation were isolated, resulting from the various propagation time of sourdoughs (Corsetti et al., 2003; De Vuyst et al., 2002).

Type I sourdough is dominated by *Lactobacillus sanfranciscensis*, as well as *Lactobacillus brevis*, *Lactobacillus plantarum*, and *Lactobacillus rossiae*. On the other hand, *Lactobacillus amylovorus*, *Lactobacillus fermentum*, *Lactobacillus pontis*, and *Lactobacillus reuteri* are common isolates from Type II sourdough (De Vuyst et al, 2009).

L. reuteri, an intestinal isolate and a stable member of sourdough (Su et al., 2012), prevails in Type II sourdough fermentations, characterized by the elevated fermentation temperatures with long incubation time (Vogel et al., 1999; De Vuyst et al., 2009). In addition, it has been established that the long fermentation time and high temperature (42°C) act as the selective pressure for *L. reuteri* (Meroth et al., 2003; Su et al., 2011). *L. reuteri* is well characterized in fermented food products as well as in rodents' intestinal tract. A study on the competitiveness of *L. reuteri* in sourdough revealed that the competitiveness of sourdough isolates was no less and even higher than the robustness of rodent intestinal isolates, while the selective pressure appears to be distinctive between the two habitats (Zheng et al., 2015b).

Modern bread production employs sourdough largely for the purpose of bread quality improvement, as well as additives replacement; therefore sourdough fermentation evolves to cater the functional requirements (Gänzle, 2014).

1.2 Glutamine metabolism in sourdough fermentation and flavor impact

Type II sourdough fermentation takes place at elevated fermentation temperature (35 – 45°C) and/or with long fermentation time. This condition results in proteolysis, which provides the substrates for microbial growth during fermentation and the resulting peptides and amino acids contribute substantially to the flavour of sourdough bread (Vermeulen et al., 2007).

Glutamine is the most abundant amino acid of wheat proteins and proteolysis of wheat proteins liberates high levels of glutamine (Gänzle et al., 2007). Glutamine is converted to glutamate or γ-aminobutyrate (GABA) by *L. reuteri* and other sourdough lactobacilli. Glutamine shows extremely rapid cellular turnover rates and its metabolites have important functional influence on

human physiology as well; they are essential for the optimal growth of mammalian cells in culture (Darmaun et al., 1986). In addition, glutamine plays a crucial role in the biosynthesis of nucleotide, amino-sugar and protein, as well as the glutathione homeostasis, as a metabolic precursor (Neu et al. 1996); also it is a source of oxidative energy (Tapiero et al., 2002).

Wheat proteins are low in glutamic acid, a primary amino acid responsible of imparting a delicious taste in fermented foods (Rundlett & Armstrong, 1994). Glutamate is converted from glutamine by glutaminase activity; it is commonly used to enhance the savory flavors, which occurs naturally in proteinaceous foods such as meats and seafood, as well as fermented foods such as soy sauce, cheese and sourdough bread (Jinap at al., 2010; Siragusa et al., 2007; Zhao et al., 2015). Characterized glutamate taste, named umami ("savory taste"), is not shared by glutamine (Tapiero et al., 2002).

The threshold of sodium glutamate (1g/3000 mL) is much lower than that of sucrose (1g/200mL) or salt (1g/400mL), which provides the intense sensation of "umami" taste (Ikeda, 2002). Aside from providing the "umami" taste, glutamate enhances the perception of sweetness and saltiness and decreases the sensation of sourness and bitterness. It has been suggested the compensative relation between glutamate salt and NaCl could potentially be used for lowering sodium intake (Baryłko -Pikielna et al., 2007).

Glutamine deamination accumulates the umami-tasting glutamic acid, which makes it possible for salt reduced sourdough bread and study has showed that the decrease of NaCl did not compromise the taste or texture (Zhao et al., 2015); In addition, this conversion plays a significant role in inter-conversion of glutamate to α-ketoglutarate, an integral component of the citric acid cycle and a crucial amino acceptor in the transamination reaction of amino acids (Tanous et al., 2005; Gänzle et al., 2007).

GABA has positive effects on human health (Mazzoli et al., 2010). It is the most widely distributed neurotransmitter in the vertebrate central nervous system that has potential antidepressant effect (Ko et al., 2013) as well as the potential of lowering blood pressure in mild hypertensive patients (Inoue et al. 2003). GABA is also crucial in mediating cholesterol level, with the potential of lowering risks of diabetes; it is a well-established inhibitory neurotransmitter, and its deficiency is associated with Huntington's chorea, Parkinson's disease,

cognitive impairment and Huntington's chorea (Shimada et al., 2009; Soltani et al., 2011). GABA, synthesized by lactic acid bacteria, was used for the production of functional fermented food. Known for their biosynthesis of GABA, *Lactobacillus plantarum* C48 and *Lactococcus lactis* strains were selected for the fermentation of GABA enriched sourdough bread; the functional sourdough bread exhibits higher concentration of free amino acids, GABA, phenolic compounds, elevated antioxidant activity and lower *in vitro* starch hydrolysis rate when compared to conventional yeast fermented bread (Coda et al., 2010). GABA-enriched cereal foods were also found such as rice germ soaked in water, germinated brown rice (Oh, 2003), and germinated wheat (Nagaoka, 2005) and red-mold rice containing *Monascus* fungus (Rhyu et al., 2000).

1.3 Glutaminase and glutamate decarboxylase in *Lactobacillus reuteri*

Glutaminase (glutamine amidohydrolase EC 3.5.1.2) catalyzes the hydrolysis of glutamine to glutamic acid and ammonia. It is a key enzyme to the formation of the savoury "umami" flavour of fermented food.

Glutaminases have diversified microbial sources, well distributed among prokaryotes and eukaryotes including bacteria, yeast and fungi, with various fermentation conditions (Nandakumar et al., 2003). The variation of environments determines that glutaminases have a wide range of optimal pH, temperature and metabolic specificity (Woraharn et al., 2014).

Despite the fact that glutaminase activity and applications were explored extensively (Nandakumar et al., 2003), the genetic determinants in *Lactobacillus* spp for glutamine metabolism were not clear and not fully understood (Teixeira et al., 2014). Glutaminase activity from crude cellular extracts of *Lactobacillus rhamnosus* was characterized on biochemical level (Weingand-Ziadé et al., 2003). *Lactobacillus sanfranciscensis* and *L. reuteri*, as well as *Lactococcus lactis* subsp. *cremoris* B78, *Lactobacillus paracasei* subsp. *paracasei* converted glutamine to glutamate, confirming the glutaminase activity (Vermeulen et al., 2007).

The deamination ability of glutaminase, as a flavour enhancer, draws attention in the food industry. Fermentation organisms with glutaminase activity are widely used in food products. For example, glutamic acid is the main contributor to the unique flavor of fermented Japanese soy sauce (concentrations of 100.6 ~ 104.0 mmol/L) (Kaneko et al., 2011). Not only does the

activity of glutaminase contribute to soy sauce fermentation (Nandakumar et al., 2003), it also provides the savory taste of "sufu" — a fermented Chinese soybean curd (Han et al., 2001). The free glutamate level ranges from 0.21 to 1.2 g/100g of a variety of cheeses, contributing simultaneously to the flavour of cheeses (Giacometti, 1979). In addition, the taste active glutamate enhances the flavour of sourdough bread fermented with *L. reuteri* (Zhao et al., 2015).

Glutamic acid decarboxylase (GAD, EC 4.4.1.15) is an enzyme that decarboxylate glutamic acid and produces γ-aminobutyric acid (GABA), using pyridoxal as cofactor (Krnjević, 1974). GAD is widely distributed and has been confirmed to be present in many lactic acid bacteria, as well as in mammalian brain, *E. coli* and *Aspergillus* (Komatsuzaki et al., 2008). Characterization of GAD in lactobacilli such as *Lactobacillus brevis* and *Lactobacillus paracasei* has been described (Park & Oh, 2006; Komatsuzaki et al., 2008). Bacteria strains that exhibit GAD activity are varied in their ability to produce GABA and optimum parameters, such as temperature and pH. The optimal pH range for GAD is 4.2-4.7 and the gene is only expressed under acidic condition (Hiraga et al., 2008; Kim et al., 2007).

In general, lactobacilli exhibit glutaminase and glutamate decarboxylase activity in a strain-specific manner; therefore, not all lactic acid bacteria strains have the ability to convert glutamine to glutamate and then generate GABA.

Functional genes screening through the database of IMG (Integrated Microbial Genomes) shows that, *gls*1, *gls*2, *gls*3, three genes that code for three glutaminses are identified in *L. reuteri* 100-23 (Teixeira et al., 2014). Alignment of protein sequences for the three glutaminases in *L. reuteri* 100-23, Gls1, Gls2, and Gls3, showed 44%-66% homology; while alignment of protein sequences of the three glutaminases in *L. reuteri* 100-23 showed 28% - 38% of homology when compared with the glutaminases YbaS and YneH in *E. coli* W3110 and YbgJ and YlaM in *Bacillus subtilis* ATCC 23857D-5 (Brown et al., 2008; Teixeira et al., 2014).

Two *L. reuteri* strains are readily available in this project: *L.reuteri* 100-23 wildtype strain that contains both GLS and GAD enzyme activities; and *L. reuteri* Δ*gad*B generated by Su et al (2011), which is an isogenic mutant of the *L.reuteri* 100-23 that has the encoding genes for glutamate decarboxylase removed.

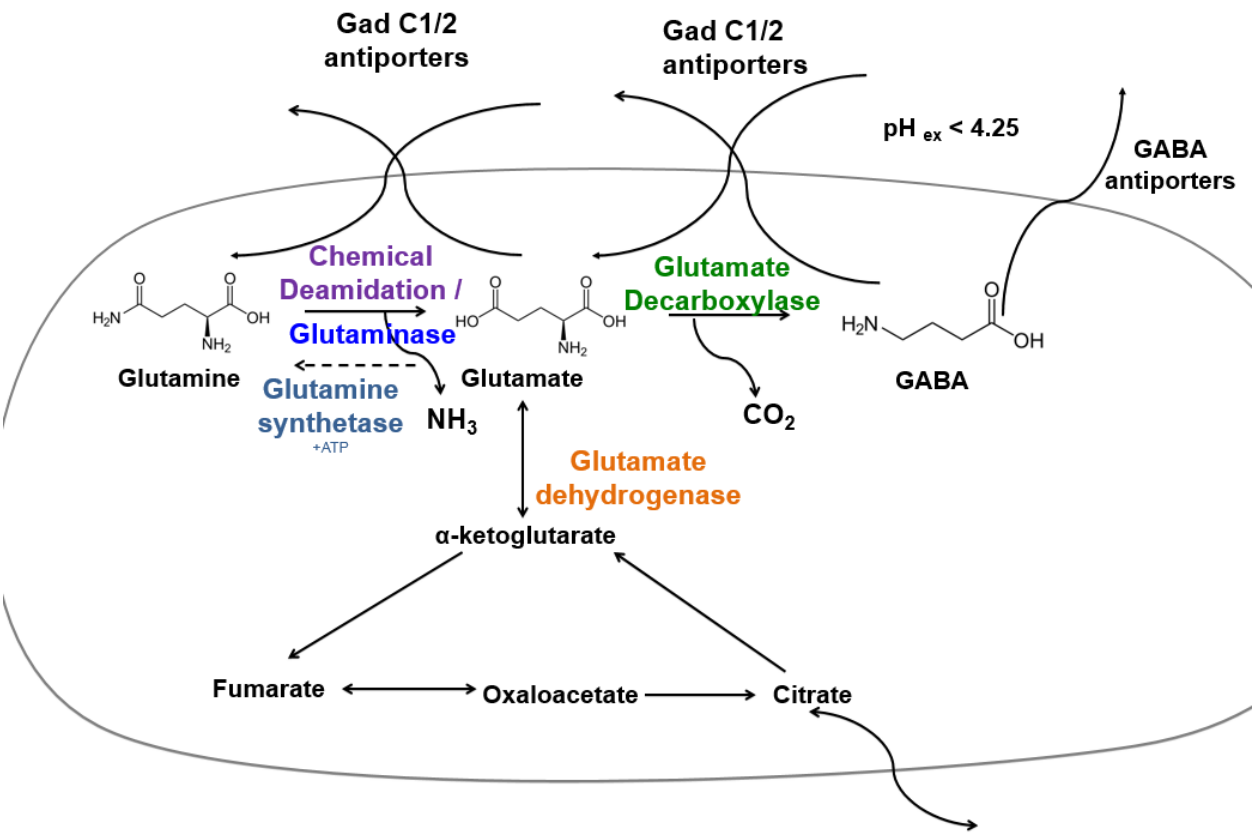

Figure 1. Proposed glutamine and glutamate metabolic pathway in *L. reuteri* 100-23

(Based on Gänzle et al., 2007; Tanous et al., 2005)

1.4 General mechanism of acid stress to *Lactobacillus reuteri*

Lactobacillus reuteri is a rodent isolate that is acid tolerant owing to the adaptation to the gastric acid and forestomach environment, therefore pH homeostasis is crucial for cell. When the cells encounter a rapid shift of the extracellular pH, the intracellular pH changes accordingly to keep the pH gradient fluctuation as minimal as possible (Cook & Russell, 1994; Siegumfeldt et al., 2000). Comparison between lactic acid bacteria strains with varied acid-tolerant abilities showed that intracellular pH regulation is in accordance with acid tolerance, among which the intracellular pH of *Lactobacillus delbrueckii* subsp. *bulgaricus* dropped faster than that of

Streptococcus thermophiles when extracellular pH had a sudden decrease (Siegumfeldt et al., 2000). Proteins that were identified in *Lactobacillus reuteri* indicated that the reaction to acid stress are related to multiple divisions, including energy metabolism, coenzyme metabolism, amino acid transportation / metabolism and etc. (Lee et al., 2008).

From the energy conservation point of view, maintaining a steady pH gradient over translocating proton is preferred by anaerobic lactobacilli spp. (Kobayashi et al., 1986). When encounters a sudden acid stress, gene screening demonstrated that 83 genes showed changes in terms of expression in *Lactobacillus reuteri* ATCC 55730, particularly the ATPase encoding genes (Wall et al., 2007).

1.5 Amino acid based mechanisms of acid resistance

The pH in Type I and II sourdoughs ranges from 3.5 to 4.3 in wheat sourdough (Thiele et al, 2002; Stromeck et al, 2011). Therefore, *L. reuteri* in type II sourdough could be exposed to a pH of 3.5-4.3 for several days (Vogel et al., 1999). The accumulating lactic acid, acetic acid and other organic acid pose great pressure on survival under acidic conditions.

Acid resistance in bacteria is a complex mechanism that is associated with physiological adaptation and metabolic pathways. Urease catalyzed urea hyrolysis in rodent isolate *L. reuteri* 100-23 is associated with acid tolerance (Wilson et al., 2014) but urea is irrelevant in sourdough fermentation (Zheng et al., 2015a).

1.5.1 Amino acid decarboxylases

Among the several acid resistance systems proposed, one of them involves the conversion of glutamate to GABA by glutamate decarboxylase. The glutamate decarboxylase (GAD) activity has been explored and suggested to play a crucial part of acid resistance in both Gram-positive and Gram-negative bacteria.

The expression of GAD (glutamate decarboxylase) is essential to *Listeria monocytogenes* for the survival in gastric fluid (Cotter et al, 2001). In *E. coli,* an amino acid antiporter GadC, which exchanges extra-cellular L-glutamate with intracellular γ-aminobutyric acid, and the glutamate decarboxylases GadA and GadB that catalyze the reaction of glutamate to GABA, helps the pathogen to cope with acid stress (Foster, 2004; Lu et al., 2013). Nonetheless, the GAD– GABA

antiporter system contributed profoundly to the acid resistance of *Lactobacillus brevis* NCL912, a high GABA-producing strain isolated from fermented vegetables (Huang et al., 2012); Besides, *Lactococcus lactis* subsp. lactis has been confirmed to harbor one GAD gene and it is associated with acid resistance (Nomura et al., 1999); Moreover, in *L. reuteri*, it has been demonstrated that GAD activity contributes to acid resistance and enhances the fitness in sourdough fermentations (Su et al., 2011).

Decarboxylation is the reaction that releases carbon dioxide from the substrate. Aside from GAD, amino acid decarboxylases also play a crucial role in pH homeostasis and acid resistance of *Lactobacillus* spp.

One example is histidine decarboxylase (Hdc), which catalyzes the conversion of histidine to histamine. It has been established that *Lactobacillus buchneri* ST2A maintains intracellular pH involving the histidine decarboxylase and an antiport mechanism that exchanges histidine and histamine (Molenaar et al., 1993). In addition, some lactobacilli strains exhibit tyrosine decarboxylase (TyrDC) activity that produces tyramine (Zheng et al., 2015a). The acid resistance mechanism of *Lactobacillus brevis* was associated with tyrosine decarboxylation operon (Lucas et al., 2007). Last but not least, ornithine decarboxylase (ODC), which catalyzes the formation of putrescine, appears to be the most frequent decarboxylase among the *Lactobacillus* spp. screened (Zheng et al., 2015a). *Lactobacillus saerimneri* 30a, a horse stomach isolate, showed distinctive system that contains lysine/ ornithine decarboxylases with a lysine/cadaverine and ornithine/putrescine transporter (Romano et al., 2013). Furthermore, the acid resistance was enhanced by ODC in three lactic acid bacteria strains, including *Lactobacillus brevis*, *Oenococcus oeni* and *Lactobacillus saerimneri* (Romano et al., 2014).

1.5.2 Amino acid deiminases

Another acid resistance system is linked to arginine metabolism. In lactic acid bacteria, it is achieved through the arginine deiminase (ADI) pathway, in which the acidic pressure is relieved by intracellular consumption of protons and the production of ATP (Konings, 2002). One mole of arginine converting to ornithine produces 1 mole of ATP and 2 moles of ammonia, with the consumption of 2 moles of protons as shown in *Lactobacillus sakei* and *Lactococcus lactis* MG1363 (Champomier-Verges et al, 1999; Budin-Verneuil et al, 2006). For *Streptococcus rattus*

FA-1 and *Streptococcus mutans* UA159，agmatine deiminase system (AgDI), an analog of the arginine deiminase system provides the competitive fitness through the production of ATP and ammonia to increase the pH (Griswold et al., 2009). In *Lactobacillus brevis*, a functional agmatine deiminase with high specificity was identified; acid resistance is attributed to a genomic region that are comprised of the agmatine deiminase and tyrosine catabolic pathways (Lucas et al., 2007). What is more, during sourdough fermentation, arginine is released by cereal proteinase and converted by ADI to citrulline, and further converted to ornithine by ornithine transcarbamoylase (OTC) and carbamate kinase (CK) (Rollan et al, 2003; Thiele et al. 2002).

1.5.3 Glutaminase and acid stress

As mentioned before, glutamine is converted to glutamate by glutaminase and produces ammonia at the same time. When the cells encounter acid stress, intracellular pH is increased resulting from the proton- neutralizing free ammonia (Lu et al., 2013).

The glutaminase enzyme activity in *Lactobacillus rhamnosus* is characterized in crude cellular extracts, suggesting a high salt and thermal-tolerant glutaminase (Weingand- Ziadé et al. 2003). Brown and others has identified and characterized the glutaminases YbaS and YneH from *Escherichia coli* and YlaM and YbgJ from *Bacillus subtilis*, and indicates that *E. coli* glutaminases are responsible for extracellular glutamine metabolism and also associated with acid defense (Brown et al., 2008). Furthermore, Lu and his team identified a novel acid resistance system that is composed of the glutaminase YbaS and the amino acid antiporter GadC in *E. coli* and it is active when sufficient glutamine available in acidic environment (under pH 6.0) (Lu et al., 2013).

L. reuteri is a rodent isolate that colonizes the forestomach, but it is also found throughout the digestive tract, including the cecum, in which the pH values are closer to neutral. For rodent-derived strain *L. reuteri* 100-23, genes contributing to colonization the forestomach of mice and adaptations of gastric acidic environment were determined, among which the GAD was supplementary and the urease cluster was the main defense factor in coping with acid stress (Krumbeck et al., 2015). Another study screened the adaptation by comparative genomics of 16 strains of *L. reuteri* and results indicated that the competitiveness of sourdough isolates was higher than the rodent intestinal isolates (Zheng et al., 2015b).

Glutaminase activity is strain specific in lactic acid bacteria. In *L. reuteri* 100-23, there are *gls*1, *gls*2, and *gls*3 that code for the three functional glutaminases. It is reported that glutamine has protective effect on *L. reuteri* survival at pH 2.5, based on the experiment established with *L. reuteri* 100-23 (Teixeira et al., 2014). However, the function of glutaminase in acid-tolerant *Lactobaillus* remains unclear.

With the comparison of the triple-knockdown glutaminase mutant and the wild type *L. reuteri* 100-23, it is possible to find out whether glutamine conversion to glutamate by glutaminase activity improves the survival of *L. reuteri* in acidic conditions independent of other amino acids conversion.

1.6 Research objectives

L. reuteri 100-23, a rodent fore-stomach isolate (Wesney & Tannock, 1979), also dominates in type II sourdough. With the ability to produce glutamate or/and GABA from glutamine, *L.reuteri* plays a significant role in sourdough fermentation in terms of flavour impact, as well as the formation of a principle neurotransmitter-GABA. Glutamate decarboxylase is well characterized in *L. reuteri* 100-23, and the enzyme improves the cell survival under acid pressure and enhances the fitness of *L. reuteri* in sourdough fermentation (Su et al., 2011).

Glutaminase is known to produce the "umami" flavor compound, glutamic acid; however, its function in terms of acid resistance in lactobacilli has not been fully investigated and established, and comprehensive understanding of the glutaminase activity of lactobacilli to glutamine metabolism in sourdough fermentation is lacking.

In particular, *L.reuteri* 100-23*,* a strain possesses three functional glutaminases. This study employed *L. reuteri* 100-23 and a derivative strain with a truncated glutamate decarboxylase (*L. reuteri* 100-23Δ*gad*B). To determine the role of glutaminase activity in acid resistance and influence on sourdough bread flavour, the three glutaminases of *L. reuteri* 100-23 were successively truncated by an unmarked deletion method to generate *L. reuteri* 100-23 Δ*gls*1-2-3.

The hypothesis is that with the disruption of glutaminase activity, the fitness of *L. reuteri* 100-23 Δ*gls*1-2-3 under acid stress will be impaired; and there will be no accumulation of the glutamate in fermentation that leaves an impact on the taste of sourdough bread produced with *L. reuteri* 100-23 Δ*gls*1-2-3.

This master project is designed to investigate the role of glutaminase activity in *L. reuteri* 100-23 throughout sourdough fermentation, to understand whether or not the glutamine conversion to glutamate by glutaminase activity improves the survival of *L. reuteri* in acidic conditions independent of other amino acids conversion.

The objectives of this study are as follows:

1. Construction of a triple-deletion *L. reuteri* 100-23 mutant by a double crossover method;

2. Assess the contribution of glutaminase in *L. reuteri* to acid resistance in buffer and to growth in sourdough.

3. Quantification of the *L. reuteri* glutaminase-relevant metabolites during sourdough fermentation by HPLC;

4. Determine the influence of glutamine metabolism on bread taste, by conducting consumer sensory evaluation of sourdough bread produced with *L. reuteri* 100-23 Δ*gad*B, *L. reuteri* 100-23 Δ*gls*1-2-3 and wild-type strain.

2 MATERIALS AND METHODS

2.1 Strains, plasmids and culture conditions

All bacterial strains and plasmids used in this study are listed in Table 1; *L. reuteri* 100-23, *L. reuteri* 100-23 Δ*gls*1, *L. reuteri* 100-23 Δ*gls*2 and *L. reuteri* 100-23 Δ*gls*3 were grown anaerobically in modified MRS (mMRS) broth at 37°C overnight. These mutants and corresponding plasmids were generated by Marcia Su (2011) and were initially assessed by Chonggong Zhang; phenotype was not observed due to the three glutaminases in *L. reuteri* 100-23 and they were not used in publications. Each liter of mMRS broth contained 10 g tryptone, 10 g maltose, 5 g glucose, 5 g fructose, 5 g beef extract, 5 g yeast extract, 4.0 g potassium phosphate dibasic, 2.6 g potassium phosphate monobasic, 2 g tri-ammonium citrate, 0.5 g L-cysteine, 0.2 g magnesium sulfate, 0.05 g manganese sulfate, 1 g Tween 80, and 1 mL of a vitamin mixture containing 0.2 g each of vitamins B1, B2, B6, and B12, folic acid, and pantothenic acid.

Plasmid host *E. coli* JM 109 pJRS pKO-*gls*1-AB, pKO-*gls*2-AB, and pKO-*gls*3-AB were grown aerobically in Luria-Bertani (LB) with 500 μg/mL of erythromycin. Erythromycin (500 mg/L) was added to LB for selecting antibiotic-resistant *E. coli*. Each liter of LB contained 10 g tryptone, 5 g yeast extract, and 10 g NaCl in deionized water.

2.2 Construction of *L. reuteri* 100-23 mutants

To understand and determine the contribution of glutaminase activity to acid resistance and the following impact on sourdough fermentation, a deletion mutant of *L. reuteri* was necessary for the comparison with the wild type strain.

Functional genes screening through the database of IMG (Integrated Microbial Genomes) showed three genes, *gls*1, *gls*2, *gls*3, are identified in *L. reuteri* 100-23 that code for glutaminses (Teixeira et al., 2014). To achieve the desired mutant that does not have the ability of converting glutamine to glutamate, the three coding genes need to be disrupted. Therefore, a three-time deletion mutant *L. reuteri* 100-23 *Δgls*1-2-3 construction is explained in detail in the following.

The three putative glutaminase (*gls*) genes in *L. reuteri* 100-23 were truncated using the double-crossover method. The plasmids and primers used are listed in Tables 1 and 2. Gene deletions in all mutant strains were verified by sequencing (MacrogenUSA, Rockville, MD).

2.2.1 Overview of mutants generation: Mechanism of Double Cross-over Mutagenesis

Shuttle vector pJRS233, constructed to incorporate the disrupted target gene region, was introduced into the electro-competent wild-type strain by electroporation.

The transformants were grown on agar plate with antibiotic selection under permissive temperature (37°C) first; then colonies were picked and incubated in broth with antibiotic pressure at 37°C overnight; integration of the plasmid into the wide-type chromosome was achieved by incubating cells at the non-permissive temperature under antibiotic selection. Therefore, subculture was transferred into broth with antibiotic under non-permissive temperature (42°C) for 30~40 generations, to select the first cross-over product: plasmid-wildtype integrates. Colony PCR was applied to check the transformation and to confirm the integrates. Under UV light after electrophoresis, the integrates would have two bands (long-wildtype and short-truncated gene) on the agarose gel.

Then integrates were then incubated in broth without antibiotic under permissive temperature (37°C) again, for about ~100 generations (~10times, every 12 hours). Cell culture was patch plated onto agar plates with and without antibiotic; colonies that only grow on plate without antibiotic were picked; colony PCR was performed to select the deletion mutant. PCR products from mutant strains would yield a short band (low amplicon-truncated gene) on the agarose gel.

2.2.2 *L. reuteri* 100-23 mutants generation

L. reuteri 100-23 Δ*gls*2-3

Plasmid DNA isolation was performed with GeneJET plasmid Miniprep kit (Thermo scientific), with 1.5mL overnight cell culture in LB broth with 500 μg/mL erythromycin. 1 ng ~ 3 μg of purified plasmid DNA in 5 μl of ddH_2O was gently mixed with 100 μl of competent cells, on ice. Electro-competent lactic acid bacteria cells were prepared with sucrose-glycerol solution and described below.

Frozen stock of *L. reuteri* 100-23 Δ*gls*2 culture was streaked on mMRS plate and anaerobically incubated at 37°C for 48 hours; a single colony was picked and transferred into 7mL of mMRS broth, grown at 37°C overnight. Cell culture were inoculated into pre-warmed (37°C) mMRS broth to obtain 40mL cell culture with the initial $OD_{600} \cong 0.1$, followed by incubation at 37°C for about 3~5 hours to achieve an optimal $OD_{600} \cong 0.55$~0.65; then cultures were chilled on ice for 10 minutes to slow down the cell activity. All the following steps were handled on ice with caution, which means mixing does not involve vortex but gentle pipetting. Cells were harvested by centrifugation at 4°C for 5 minutes, washed gently in 20mL chilled ddH_2O twice and centrifuged 4°C, for 5 minutes each time; cells then were washed gently with 20mL chilled 0.5M sucrose with 10% V/V glycerol solution and harvested by centrifugation at 4°C, for 5minutes. Last, harvested cell pellets were re-suspended in ~350 μL of the chilled sucrose-glycerol solution and aliquots of 100 μL electro-competent cells were used for electroporation.

Then the mixture was transferred into to a pre-chilled (0°C) cuvette (0.2 cm gap) and pJRS pKO-*gls*3-AB DNA was integrated into the competent *L. reuteri* 100-23 Δ*gls*2 cells at 2.5 kV (12.5 kV/cm), 25 μF capacitance, and 400 Ω resistance. 1 mL of pre-warmed (37°C) mMRS medium was added to the cuvette immediately and the electroporated cells were incubated at 37°C about 2.5 - 3hours, for expression of the antibiotic resistance genes.

The vector pJRS233 carries a temperature-sensitive replication origin pSC101 and an erythromycin-resistance gene, and the increase of growth temperature and the addition of erythromycin promote the integration of pKO-*gls*3-AB into the chromosome of erythromycin-sensitive *L. reuteri* 100-23 Δ*gls*2 by homologous recombination (Su, 2011).

Then 100 μl undiluted electroporated culture was plated on mMRS+10 μg/mL erythromycin agar plate, incubated at 37°C anaerobically for ~48 hours. Colonies were picked and grown in mMRS broth +10 μg/mL erythromycin at 37°C initially and then 42°C for 30-40 generations. The later selection procedure was indicated above in the mechanism part: ~100 generations subculture in mMRS broth and patch-plating selection. Polymerase chain reaction (PCR) amplifications for screening purposes were performed with Taq DNA Polymerase (Invitrogen, Burlington, Canada).

Both cross-over products were confirmed by colony PCR with the primer gls3-F and gls3-R; and the gene deletion of *gls*3 in *L. reuteri* 100-23 Δ*gls*2-3 was verified by DNA sequencing (MacrogenUSA, Rockville, MD).

***L. reuteri* 100-23 Δ*gls*1-2-3**

The gene coding for *gls*1 in *L. reuteri* 100-23 was also truncated according to the deletion strategy described earlier, by introducing pJRS pKO-*gls*1-AB into *L. reuteri* 100-23 Δ*gls*2-3. Again, both cross-over products were confirmed by colony PCR with the primer gls1-F and gls1-R; and the gene deletion of *gls1* in *L. reuteri* 100-23 Δ*gls*1-2-3 was verified by DNA sequencing (MacrogenUSA, Rockville, MD).

TABLE 1. Strains and Plasmids Used in this study

Strain or Plasmid	Description	Source
Strains		
Lactobacillus reuteri 100-23	Rodent isolate; wild type strain	Wesney et al., 1979
Escherichia coli JM109	Cloning host for pGEMTeasy- and pJRS233-derivative plasmids	Su et al., unpublished
L. reuteri 100-23 Δ*gls*1 (UA 3395)	Wild-type strain isogenic mutant with *gls*1 deletion	Su et al., unpublished
L. reuteri 100-23 Δ*gls*2 (UA 3396)	Wild-type strain isogenic mutant with *gls*2 deletion	Su et al., unpublished
L. reuteri 100-23 Δ*gls*3 (UA 3397)	Wild-type strain isogenic mutant with *gls*3 deletion	Su et al., unpublished
L. reuteri 100-23 Δ*gad*B	Wild-type strain derivative with a deletion in *gad*B	Su et al., unpublished
L. reuteri 100-23 Δ*gls*2-3	Wild-type strain isogenic mutant with *gls*2 and *gls*3 deletions	This study
L. reuteri 100-23 *Δgls*1-2-3	Wild-type strain isogenic mutant with *gls*1, *gls*2 and *gls*3 deletions	This study
Plasmids		
E. coli JM 109 pJRS pKO-*gls*1-AB	Plasmid for the disruption of 2500069653 (or *gls*1) in *L. reuteri* 100-23	Su et al., unpublished
E. coli JM 109 pJRS pKO-*gls*2-AB	Plasmid for the disruption of 2500070771 (or *gls*2) in *L. reuteri* 100-23	Su et al., unpublished
E. coli JM 109 pJRS pKO-*gls*3-AB	Plasmid for the disruption of 2500071323 (or *gls*3) in *L. reuteri* 100-23	Su et al., unpublished

TABLE 2. Primers used to generate derivatives of *L. reuteri* 100-23 by double-crossover mutagenesis

Primer Name	Sequence 5' to 3'	Reference
gls1-F	GCCAAATATCTGCTGATCG	*gls*1
gls1-R	AACAGCGTTTGTTCCAA	> In NCBI GenBank database, it is coded by AAPZ02000001.1:333174..334094 Version: AAPZ02000001.1 GI:194453319
gls1-out-F	GCAGTTGAAGAGCAAGTC	
gls1-out-R	AGCAGCCTCGCTTGA	
gls2-F	CCCGCTCTTATTTAGGAATGT	*gls*2
gls2-R	TACTATCCATATCGGTTGGGC	> In NCBI GenBank database, it is coded by AAPZ02000001.1:1455778..1456692 Version: AAPZ02000001.1 GI:194453319
gls2-out-F	TGCAAAGGTCGTTGCCTTAAT	
gls2-out-R	GTCACCATCATTGAGCTTTGC	
gls3-F	CCTTTATCAACCATCAGCT	*gls*3
gls3-R	AGCTGGTGTGCTACTTT	> In NCBI GenBank database, it is coded by AAPZ02000002.1:548219..549139 Version: AAPZ02000001.1 GI:194453319
gls3-out-F	AGGTTGCTACTTCAGTAGGAA	
gls3-out-R	ATGTAACGAAACATGTTGGAG	
HRM-LAB-F	TCC TAC GGG AGG CAG CAG T	Lin & Gänzle, 2014
HRM-LAB-R	GGA CTA CCA GGG TAT CTA ATC CTG TT	

2.3 Assessment of acid resistance at pH 2.5 and pH 3.5

Frozen stock culture of *L. reuteri* 100-23, *L. reuteri* 100-23 Δ*gls1-2-3* and *L. reuteri* 100-23 Δ*gad*B were steaked onto mMRS agar plates and single colony of each strain was inoculated into mMRS broth and incubated at 37°C overnight. Cells were grown in mMRS medium at 37°C from a 1% inoculum and growth was monitored by measuring the optical density (OD) at 600 nm. They were grown to the early stationary phase of growth (14~18 h) and harvested by centrifugation, washed with pH 7.0, 50mM Na_2HPO_4 buffer and then re-suspended in 200mM potassium phosphate buffer to an OD_{600nm} of 1.0, with or without additional glutamate or glutamine at various pH level, adjusted with HCl.

To determine the contribution of amino acid metabolism by glutaminase activity in *L. reuteri* to acid resistance, a pH of 2.5 adjusted with HCl was chosen to match the gastric fluid environment and a pH of 3.5 was selected to mimic the sourdough fermentation condition.

Controls were performed in 200mM potassium phosphate buffer (pH 2.5 and 3.5); parallel experiments were conducted in 200mM potassium phosphate buffer (pH 2.5 and 3.5), supplemented with 20 mM glutamine or glutamate (Sigma-Aldrich, St. Louis, MO). The pH was readjusted with HCl to pH 2.5 or 3.5 after the addition of amino acids and buffer was filtered before the resuspension of cells.

OD_{600nm} of the above three *L. reuteri* strains in acidic buffer were monitored and the level of supplemented glutamate or glutamine was measured by HPLC.

Buffer with a pH of 2.5 treatment was incubated at 37 °C for 10 hours and pH 3.5 treatment was analyzed for 24 hours, based on previous experiments parameters (Su et al., 2011; Teixeira et al., 2014). To observe the survival of the bacteria, samples were taken for viable-cell counts at intervals, as well as for the quantification of amino acids. Samples were immediately mixed with phosphate-buffer saline (PBS, 137 mM NaCl, 2mM KCl, 10 mM Na2HPO4, and 2 mM KH_2PO_4, pH 8.0) and proper dilutions in PBS buffer of each sample were plated onto mMRS agar. Plates were incubated at 37°C for 48 h anaerobically.

L. reuteri 100-23, *L. reuteri* 100-23 Δ*gls1-2-3* and *L. reuteri* 100-23 Δ*gad*B were also incubated at pH 6.2 in 200 mM potassium phosphate buffers, with or without additional 10mM glutamate

or glutamine at 37 °C for 24 hours. Supernatant of the buffer after 24-hour incubation were stored at -20°C and used for HPLC analysis later.

2.4 Sourdough fermentations and sampling

Sourdough fermentations were performed with of *L. reuteri* 100-23, *L. reuteri* 100-23 Δ*gls*2-3, *L. reuteri* 100-23 Δ*gls*1-2-3 and *L. reuteri* 100-23 Δ*gad*B respectively, to examine the effect of glutaminase activity on growth, pH, organic acid, and amino acid accumulations.

2.4.1 Sourdough fermentation

L. reuteri 100-23, 100-23 Δ*gad*B*,* 100-23 Δ*gls*2-3, 100-23 Δ*gls*1-2-3 were cultured at 37°C in mMRS broth. Cells were harvested from 10mL overnight culture by centrifugation at 4°C, for 5 minutes; washed with 10mL autoclaved tap water, and then re-suspended in 10mL autoclaved tap water.

Sourdoughs were prepared in a 50 mL screw-cap tube by mixing 10 mL of washed cell culture and 10 g of whole-wheat flour thoroughly (Rogers foods LTD., Armstrong, BC, Canada). Chemically acidified doughs were used as control, for which 10 g of whole-wheat flour were mixed with 10mL autoclaved tap water and acidified to a pH of ~4.0 (~30µL of acetic acid (100%w/w) and ~120µL lactic acid (85% w/v)) and incubated under the same conditions as the sourdoughs.

Sourdough samples were collected at 0, 24, 48, 72, and 96 h of fermentation, for cell count, pH measurement and HPLC analysis of organic acids and amino acids. The pH and the cell counts were analyzed immediately after sampling; HPLC samples were stored at -20°C and analyzed later.

2.4.2 Determination of pH and cell counts

For determination of the pH, 1 g of sourdough samples were mixed with 9mL of deionized water and the pH was measured with a glass electrode (Thermo electron corporation, Orion 4 star). Triplicate independent samples were taken at time 0, 24, 48, 72 and 96 hours of each treatment.

The 1 g of sourdough was diluted into 9mL of sterile peptone and 1% salt solution, then serial 10-fold dilutions were performed and samples were plated on mMRS agar plates for Cell counts.

Colony morphology was observed to verify the identity of the inoculum with the fermentation microflora.

2.5 Quantification of amino acids by HPLC

Cells from phosphate buffer were removed by centrifugation. The supernatant (1 vol) was mixed with deionized water (4 vol), saturated potassium borate (4 vol) and 5ug/mL β -amino-butyric acid (BABA) (1 vol). Quantification of amino acids in sourdough samples were achieved by internal standard method for calibration. BABA was the internal standard (IS) used and ethanolamine (EA) (5umol/mL - 0.2439g/100mL) as an additional internal standard for backup in the event of BABA contamination etc., although EA appears to be less stable.

Sourdough samples were lyophilized, extracted with distilled water at an extraction ratio of 1:10 (w/v) and diluted with water, potassium borate, and internal standard as described above.

Bread samples were freeze-dried, extracted with 1M HCl at the ratio of 1:10 (w/v) and diluted with water, potassium borate, and internal standard as described above.

Both buffer and sourdough samples were done in triplicate independent experiments and bread samples were collected in triplicate repeats. Amino acids were quantified after derivatization with o-phtaldialdehyde. Separation and quantification of amino acids is accomplished with an HPLC (high performance liquid chromatograph) and a Fluorichrom detector (excitation 340 nm emission 450 nm); Separations are achieved using a Supelcosil 3 micron LC-18 reverse phase column (4.6 x 150 mm; Supelco) equipped with a guard column (4.6 x 50 mm) packed with Supelco LC-18 reverse phase packing (20 - 40 μm) (modified based on Sedgwick et al., 1991).

2.6 Sourdough bread sensory evaluation

Research ethics approval was obtained by the University of Alberta Research Ethics Board, after reviewing this sensory study according to the ethical guidelines. The most abundant amino acid in wheat proteins is glutamine. Glutamine is converted to glutamate or γ-aminobutyrate by *Lactobacillus reuteri* and other sourdough lactobacilli. Sensory evaluation was designed to exam the impact of glutamine and glutamate metabolism on sourdough bread taste.

2.6.1 Strains and media

L. reuteri 100-23, 100-23 Δ*gad*B*,* 100-23 Δ*gls*1-2-3 were cultured at 37°C mMRS medium. 1% (v/v) of each strain liquid culture inoculum was added into 5% (w/v) food-grade malt solution respectively, using CBW Munich malt extract (Briess Malt and Ingredients, Cgilton, WI, U.S.A.)

Malt culture was incubated at 37°C in food-grade laboratory overnight for sourdough preparation.

2.6.2 Sourdough fermentation

To determine the influence of glutamine metabolism on bread taste, sourdough was fermented with *L. reuteri* 100-23, 100-23 Δ*gad*B*,* or 100-23 Δ*gls*1-2-3*,* and bread produced with these sourdoughs was evaluated in triangle tests with 41 untrained panelists.

Sourdough preparation and fermentation were conducted in a food-grade laboratory, in order to comply with the purpose of the sensory evaluation. 10g of whole wheat flour (Rogers foods LTD., Armstrong, BC, Canada) was mixed with 10mL of the overnight malt strain culture, incubated for 14~18 hours at 37°C. Sourdough was prepared by inoculating the 20g starter dough into 90g of whole wheat flour and 90mL of tap water to achieve a dough yield of 200, and incubated at 37°C with an initial cell count of $1 \pm 0.5 \times 10^8$ CFU g-1. Doughs were fermented with *L. reuteri* 100-23, 100-23 Δ*gad*B, 100-23 Δ*gls*1-2-3 separately, and samples were collected after 96 h of fermentation at 37°C. Sourdoughs were freeze-dried and flour was used for subsequent bread baking.

Cell counts and pH of the sourdough were monitored every 24 hours; organic acids, amino acids were measured by HPLC and HRM-qPCR was performed at 96 hours for quality control.

2.6.3 Monitoring microbiota during sourdough fermentation

High resolution melting (HRM) analysis is a quick and reliable technique to verify variations in nucleic acid sequence. The basic principle of HRM is to monitor and detect the differences in melting curves during real-time PCR, together with DNA binding dyes (Thermo fisher scientific-HRM).

Together with cell morphology observation and organic acid analysis, HRM-PCR is the last checkpoint to make sure that the sourdough is fermented with the corresponding *L. reuteri* strain, rather than being out-compete or contaminated by other *Lactobacillus* spp.

Sourdough samples, scraped from the container after 96 hours of incubation, were thoroughly mixed with distilled water (1:10 w/v) by vortex. The mixture then was centrifuged for 3 min to remove the solids; and the supernatant was again centrifuged for 10 min and cells pellets were collected.

Cell DNA extraction was performed with the DNeasy Blood & Tissue Kit (Qiagen, USA). Universal primers, HRM-LAB listed in Table 2, were used for targeting the 16s rRNA genes. Type-it HRM PCR kit (Qiagen, USA) was used and the PCR (Rotor-Gene Q, Qiagen, USA) set-up is as follows, 5 min at 95 °C for denaturation, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and extension at 72 °C for 10 s. And the temperature went from 65 °C to 90 °C at 0.1 °C/step with 2s holding time at each step at the end (Lin & Gänzle, 2014). Each strain of bacteria has a unique melting temperature (MT) and MT for *L. reuteri* is 86.0°C~86.5°C.

2.6.4 Bread baking and sensory evaluation

Bread dough was prepared with all-purpose wheat flour (Rogers foods LTD., Armstrong, BC, Canada) 6% freeze-dried sourdough flour, 2% sugar, 2% yeast, 60% water and 2% table salt (based on 100g of flour).

Baking is performed according to the standard procedure and the protocol is attached in the appendices, together with the sensory evaluation information and consent form and demographic questionnaire.

Ingredients were mixed in a spiral kneader (Kitchen Aid K45SS, Hobart Co. Troy, OH) for 5min, speed 2 for 2 min and speed 5 for 3 min. Dough was shaped after the dough resting of 60 min and proofed for 90 min at ~30°C, and 85% humidity in a baking proofer (Res-Cor, Crescent Metal Products Inc, Cleveland, OH, USA). Bread was baked in a multi-deck oven (Bakers Pride, Lachine, QC, Canada), set at a temperature of 210°C for 25 min.

Bread was held at room temperature for cooling after baking, and wrapped and sealed in polyethylene bags prior to sensory evaluation.

Research ethics approval was obtained by the Research Ethics Board at University of Alberta, after reviewing this sensory study according to the ethical guidelines. Bread samples were prepared by removal of the bread crust and then cutting into 2cm * 2cm * 3cm cubes. Bread cube was served in a plastic cup with lids, labeled with three-digit random code. All possible sample presentation orders of the three samples were randomly assigned to each panelist to avoid positional effect. Filtered water was provided for the panel to cleanse their palate before and in-between tasting. 41 untrained panelists were recruited and successfully completed the sensory evaluation at University of Alberta AFNS. Tasting were conducted in a sensory laboratory with controlled white lighting, 22°C, 45% RH and positive pressure.

Two sets of the conventional triangle test (Lawless & Heymann, 2010) were performed to compare breads prepared with sourdough that was fermented with *L. reuteri* 100-23 or 100-23 Δ*gad*B*;* as well as between *L. reuteri*100-23 and 100-23 Δ*gls*1-2-3.

Attributes ranking of the three sourdough bread samples, employing the Friedman rank test (Lawless & Heymann, 2010), was conducted in terms of saltiness, sourness, overall taste and overall preference.

2.7 Statistical analysis

Growth curves and survival at pH 2.5 and pH 3.5 were determined in triplicate biological independent experiments and results were expressed as mean value +/- standard deviation. Data analysis was achieved with Microsoft Excel for paired two sample-T test.

Fermentations of sourdoughs and chemically acidified controls were carried out in triplicate independent experiments, and results of pH and cell count are reported as the mean +/- standard deviation.

The amino acids integration of HPLC chromatogram was processed with the software Galaxy Chromatography. Results were reported as the mean +/- standard deviation.

Basic calculation formulas for the HPLC quantification of amino acids are shown as follow:

For Standards: RRF= (amount of standard/area of standard) * (area of IS/ amount of IS)

For Samples: Amount of sample= RRF * area of sample * amount of IS/ area of IS

For sensory evaluations, significant difference of the triangle test was determined by the minimum correct identification ($P < 0.05$) (Lawless & Heymann, 2010). Data of the attributes ranking was processed with Microsoft Excel, and ANOVA test was applied to determine whether or not significance difference was detected, in regards to specific attribute among the samples by the 41 untrained participants.

3 RESULTS

3.1 Confirmation of *L. reuteri* 100-23 mutants

L. reuteri 100-23 harbors three genes that code for glutaminase, *gls*1, *gls*2, *gls*3 (Teixeira et al., 2014). Therefore, deletion of the three glutaminases is necessary for the purpose of this study.

PCR was applied to confirm the deletion mutant of targeted *gls* region, using the primer sets gls1/2/3 in Table 2. In addition, another set of primers (gls1/2/3-out, Table 2) was designed to avoid false positives, to verify glutaminase coding region deletion in the mutants. These primers were selected from the internal *gls* frame and size of the amplicons were ~500 bp. Figure 1 lists four agarose gel images, illustrating the selection of *L. reuteri* 100-23 Δ*gls*2-3 and *L. reuteri* 100-23 Δ*gls*1-2-3 after colony PCR.

After subculture in mMRS broth without antibiotic pressure for about 100 generations, excision of the plasmid from the chromosome in the integrant strain via duplicated 100-23 sequence on one side of the deletion generated the desired deletion mutant; excision via duplicated sequence on the other side of the deletion regenerated the wild-type allele in the chromosome (Wild-type).

As shown on the agarose gel images in the Figure 1, deletion mutants were expected to have a short band at ~500 bp; while wild-type was expected to have a band of ~1.5kb, indicating the size of an intact *gls* region. In addition, deletion of *gls*3 in *L. reuteri* 100-23 Δ*gls*2-3 and the gene deletion of *gls*1 in *L. reuteri* 100-23 Δ*gls*1-2-3 were verified by DNA sequencing (MacrogenUSA, Rockville, MD).

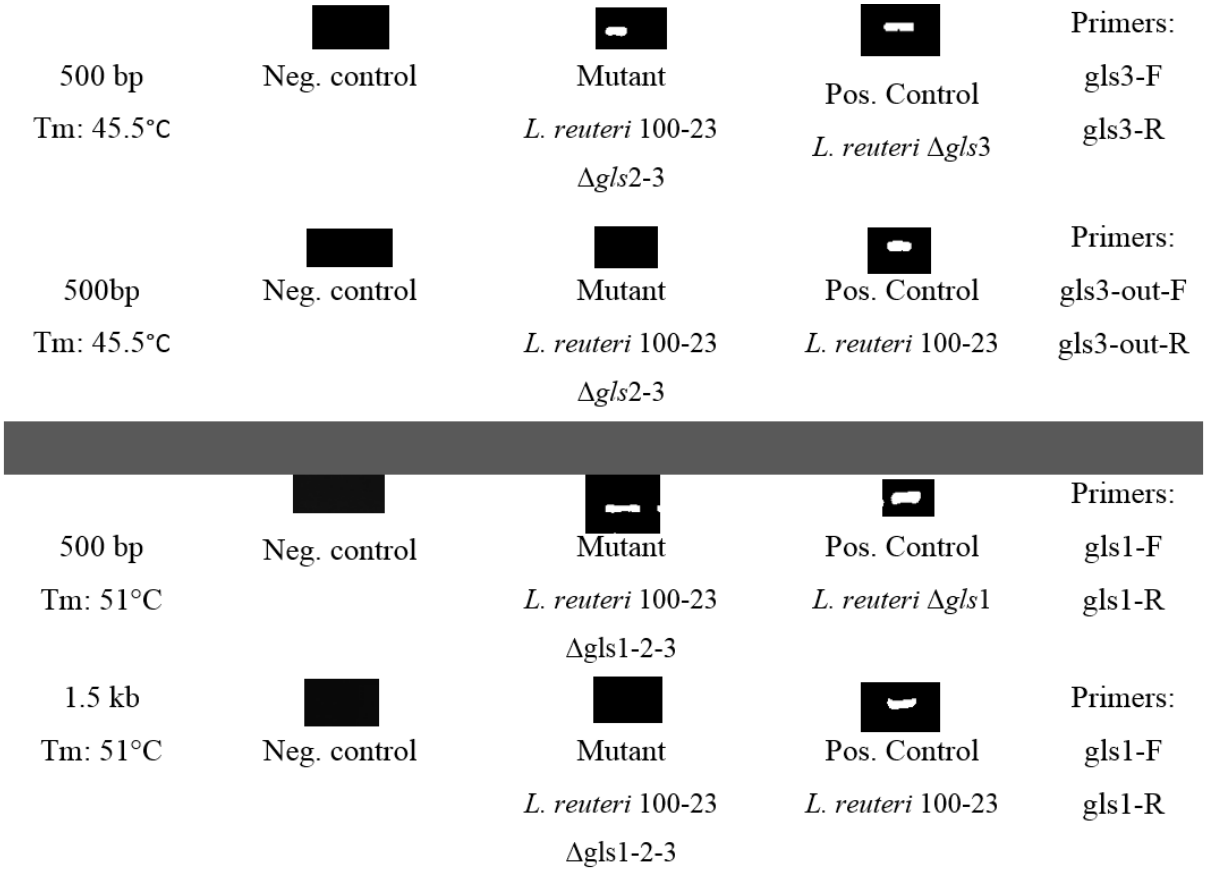

Figure 2. Confirmation of mutant by colony PCR and DNA sequencing

The PCR products of selected mutants were purified and verified by colony PCR and DNA sequencing (MacrogenUSA, Rockville, MD). *L. reuteri* 100-23 Δ*gls*2-3 and *L. reuteri* 100-23 Δ*gls*1-2-3 deletion region sequencing results are attached in appendices.

3.2 Assessment of acid resistance

Acid resistance of the wild type and the mutants were tested in buffer at pH 2.5 and 3.5, to mimic the acidic environment of the stomach (pH 2.5) and fore-stomach or sourdough fermentation (pH 3.5).

3.2.1 Survival at pH 2.5

Survival of *L. reuteri* 100-23 Δ*gad*B, *L. reuteri* 100-23 Δ*gls*1-2-3 and wild-type strain under acid stress in 200mM potassium phosphate buffer were measured. As shown in Figure 2, glutamine addition enhanced the survival of *L. reuteri* 100-23 at pH 2.5 when compared to the control that has no amino acid supplementation, but not in a significant manner. Both experiments, the control and glutamine added, had ~2.5 log reduction after 10 hours incubation in pH= 2.5 phosphate buffer.

For *L. reuteri* 100-23 Δ*gad*B, without the function of glutamate decarboxylase that metabolize glutamate, glutamate supplement did not improve the survival at pH 2.5 in buffer. After 10 hours of incubation at 37°C, both the control and glutamate treatment groups had a viable cell reduction of ~3.5 log. In contrast, additional glutamine in the buffer at pH=2.5 improved the survival of *L. reuteri* 100-23 Δ*gad*B, resulting in a ~2.5 log cell reduction; besides, there is a significant difference in terms of survival between the control and the glutamine supplementation group. This observation correlates with the previous work that examines the glutamine-based acid resistance effect (Teixeira et al., 2014), in which glutamine was protective for *L. reuteri* 100-23 Δ*gad*B against acid stress at pH 2.5 in phosphate buffer.

L. reuteri 100-23 Δ*gls*1-2-3 did not benefit from the glutamine supplementation in pH 2.5 buffers. With the deletion of all three functional glutaminses, the data in figure 2 indicated that glutamine metabolism was disrupted when compared to that in the wild type strain. However, both *L. reuteri* 100-23 and *L. reuteri* 100-23 Δ*gls*1-2-3 demonstrated that glutamate addition was the least favorable to acid resistance in pH=2.5 phosphate buffer.

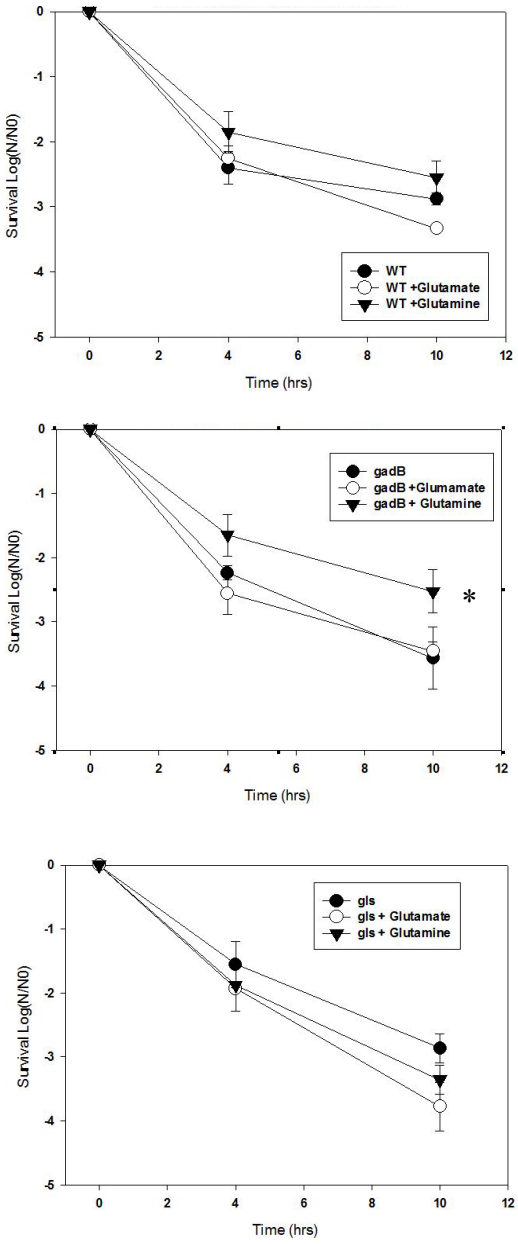

Figure 3. Survival of *L. reuteri* in pH 2.5 Phosphate Buffer. Survival of *L. reuteri* 100-23 (WT), *L. reuteri* 100-23 Δ*gad*B (gadB) and *L. reuteri* 100-23 Δ*gls*1-2-3 (gls) in 200mm pH= 2.5 phosphate buffer (●), in phosphate buffer with 20 mM glutamate (○), or in phosphate buffer with 20 mM glutamine (▼) after 10 hours of incubation at 37°C. Cell counts were plotted as log CFU/ mL (N/N0). Data are mean values of three independent experiments +/- standard deviations. * means that the two-paired T-test between the control and the treatment showed significant difference ($P<0.05$).

Therefore, it was necessary to measure the amino acid content in the buffer throughout the assessment to better understand the role of glutamine/glutamate metabolism to acid resistance.

HPLC analysis of the two amino acids supplemented samples at pH 2.5 phosphate buffer was quantified. Amino acid profiles supported that *L. reuteri* 100-23 but not *L. reuteri* 100-23 Δ*gad*B metabolizes glutamate. At t=10h, glutamate concentration dropped from 20.4 ± 1.8 mM to 15.8 ± 2.7 mM in the wild-type pH 2.5 buffer supernatant; while it remained the same for *L. reuteri* 100-23 Δ*gad*B sample. As for *L. reuteri* 100-23 Δ*gls*1-2-3, glutamate accumulation was not observed, in which glutamate concentration was 16.1 ± 1.6 mM at the start and 17.6 ± 1.0 mM after 10 hours of incubation under acid stress. This explains the adverse effect on survival of *L. reuteri* 100-23 Δ*gls*1-2-3 with glutamate addition in figure 2.

Glutamine data analysis confirmed that the triple glutaminases knock-out mutant does not have the ability to mediate the glutamine added, considering that the glutamine level of the other two strains decreased 3.1 ± 0.6 mM, whereas remained static for *L. reuteri* 100-23 Δ*gls*1-2-3. Moreover, the decrease in glutamine concentration also resolves the protective effect of glutamine on survival of *L. reuteri* 100-23 and *L. reuteri* 100-23 Δ*gad*B at pH2.5.

3.2.2 Survival at pH 3.5

Survival of *L. reuteri* 100-23 Δ*gad*B, *L. reuteri* 100-23 Δ*gls*1-2-3 and wild-type at pH 3.5 in 200 mM potassium phosphate buffers was also monitored. Instead of 10 hours observation time span, the incubation time was set to be 24 hours.

Addition of glutamine and glutamate both improved the survival of *L. reuteri* 100-23 at pH 3.5 by ~ 1.5-2 log, whereas the control group showed a reduction of ~ 4 logs after 24 hours of incubation. Moreover, there is a significant difference between the control and the glutamine or glutamate supplement group at t=24h; as a result, the wild-type benefits from the supplementation of both glutamine and glutamate at pH 3.5 buffer.

Similarly to pH 2.5 experiments, glutamate supplementation did not improve the survival of *L. reuteri* 100-23 Δ*gad*B at pH 3.5, almost 4 log cell count decrease at t=24h; while the glutamine addition had a less than 2 log reduction after 24 hours in pH 3.5 phosphate buffer. The P value between the glutamine treatment and control is 0.07 (<0.1), which indicated the trend that glutamine exhibits protective effect on cell survival. Taken both pH 2.5 and 3.5 acid resistance assessment observations into consideration, it is established that glutamate decarboxylation contributes to acid resistance in *L. reuteri* (Su et al., 2011).

Figure 3, again, showed that glutamate addition was the least favorable in pH=3.5 phosphate buffer for *L. reuteri* 100-23 Δ*gls*1-2-3. Among the three strains tested, *L. reuteri* 100-23 Δ*gls*1-2-3 had a in reductions of ~1.5-3.0 log after 24 hours treatment. In particular, the glutamate supplemented group had a reduction of ~3.0 log in terms of viable cell count.

HPLC quantification of glutamine and glutamate content of pH 3.5 buffer supernatant showed that the concentration of supplemented glutamine or glutamate decreased over the 24-hour incubation in all three strains tested; it is noticeable that *L. reuteri* 100-23 Δ*gls*1-2-3 was less efficient to metabolize glutamine at both pH 2.5 and 3.5.

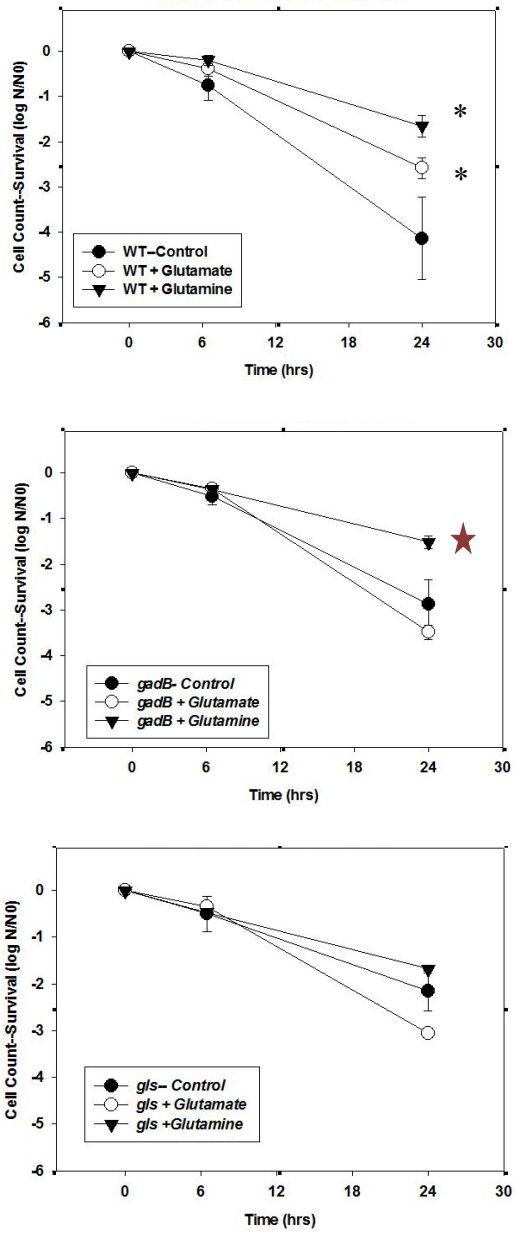

Figure 4. Survival of *L. reuteri* in pH 3.5 Phosphate Buffer. Survival of *L. reuteri* 100-23 (**WT**), *L. reuteri* 100-23 Δ*gad*B (**gadB**) *and L. reuteri* 100-23 Δ*gls*1-2-3 (**gls**) in 200mm pH= 3.5 phosphate buffer (●), in phosphate buffer with 20 mM glutamate (○), or in phosphate buffer with 20 mM glutamine (▼) after 24 hours of incubation at 37°C. Cell counts were plotted as log CFU/ mL (N/N0). Data are mean values of three independent experiments +/- standard deviations. * means that the two-paired T-test between the control and the treatment showed significant difference ($P<0.05$), and ★ represents treand of difference ($0.05<P<0.1$).

Taken together, the results demonstrate that *L. reuteri* 100-23 Δ*gad*B, *L. reuteri* 100-23 Δ*gls*1-2-3 and wild-type benefit from glutamine supplementation under acidic condition in terms of survival. Glutamine deamidation by glutaminase mediates acid resistance and protects *L. reuteri* against acid stress, independent of glutamate decarboxylation. In addition, *L. reuteri* 100-23 Δ*gls*1-2-3, based on the cell count, was less impaired under acid pressure when compared to wild-type strain on survival. Without the ability to process glutamine properly, mutant can no longer rely on *gls*-mediated glutamine metabolism to cope with acid pressure, suggesting alternative acid resistance mechanisms might be involved. Furthermore, because of the glutamate accumulation, glutamine deamidation activity was not entirely dysfunctional in *L. reuteri* 100-23 Δ*gls*1-2-3 even with the three glutaminases disrupted; however, the alternative ways of glutamine deamination do not affect acid resistance.

3.2.3 Glutamine conversion in pH 6.2 buffer

Assessment of pH 6.2 buffer for glutamine metabolism was performed. Glutamine, glutamate and GABA level were quantified with *L. reuteri* 100-23 or *L. reuteri* 100-23 Δ*gad*B or *L. reuteri* 100-23 Δ*gls*1-2-3 samples incubated at 37°C in 200mM pH 6.2 phosphate buffer with 10 mM glutamate, or with 10 mM glutamine after 24 hours. Glutamate decarboxylase activity is limited at pH 6.2 (Hiraga et al., 2008); therefore the conversion to GABA was inhibited.

Previous observation in figure 2 and 3 suggested that the conversion of glutamine to glutamate in *L. reuteri* 100-23 Δ*gls*1-2-3 was not fully disabled despite the disruption of three glutaminases. With the addition of pH 6.2 buffer assessments, the results solve the paradoxical situation observed at pH 2.5 and 3.5.

Glutamate accumulation was observed in the three strains tested, even without the glutamine added in the buffer. The concentration of glutamate in pH 6.2 buffer increased by ~ 0.13-0.41 mM after 24 hours of incubation with glutamine addition; glutamate level increased by ~ 1.9-3.1 mM while no glutamine was provided to the buffer. GABA content in pH 6.2 buffer of glutamine supplemented *L. reuteri* 100-23 and *L. reuteri* 100-23 Δ*gls*1-2-3 treatments both increased by ~ 0.02 mM.

According to figure 2 and 3, it is clear to conclude that abundant glutamine enhances acid resistance of *L. reuteri* 100-23 and *L. reuteri* 100-23 Δ*gad*B, as the survival of *L. reuteri* 100-23

and *L. reuteri* 100-23 Δ*gad*B is favored by up to ~3 logs CFU/mL when 20mM glutamine was added to the buffer. The buffer system is a simple model for monitoring bacteria survival under acidic stress, since the there was only one substrate provided in each treatment. Considering the glutamine concentration change throughout the incubation span, it can be concluded that glutamine deamination by *gls* activity under acidic environment is beneficial to *L. reuteri* 100-23 survival.

3.3 Growth during sourdough fermentation

Sourdough fermentation with *L. reuteri* 100-23 or its isogenic mutants were carried out with whole-wheat flour at 37C for 96 hours. Cell counts and pH were measured every 24 hours throughout the fermentation.

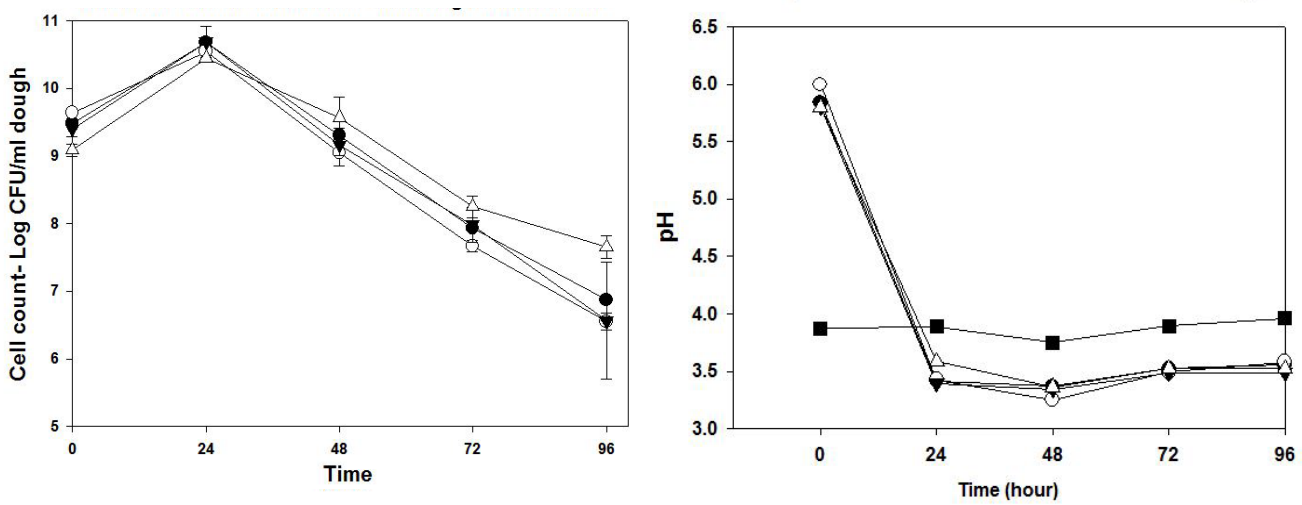

Figure 5. Cell counts and pH measurement of whole wheat sourdough fermented with *L. reuteri* 100-23 (●), *L. reuteri* 100-23 Δ*gad*B (○), *L. reuteri* 100-23 Δ*gls* 2-3 (▼), *or L. reuteri* 100-23 Δ*gls*1-2-3 (Δ), chemically acidified sourdough (■) respectively over 96 h. Symbols indicate means +/-standard deviation from triplicate determinations.

Sourdough samples had an initial inoculum of 9-9.6 log CFU/mL and the growth patterns of wild-type and mutant strains in sourdough were identical, reaching their peak cell counts of ~10.5 log CFU/mL at their first measuring time point, t=24 hours. Cell count for the chemical acidified sourdough was below the detection limit. As for pH, chemical acidified sourdough maintained a pH of 3.8-4.0 during fermentation; and other treatment groups had a pH drop from ~6.0 to ~3.5 after 24 hours of anaerobic fermentation at 37°C.

Cell counts started to decrease after the first 24 hours gradually and at the end of the fermentation; and pH remained consistent after the first 24 hours of fermentation, maintaining at ~pH 3.5.

3.4 Metabolism of glutamine in sourdough

3.4.1 Glutamine, glutamate and GABA in sourdough

The metabolism of glutamine and glutamate by *L. reuteri* 100-23 Δ*gad*B, *L. reuteri* 100-23 Δ*gls* 2-3, *L. reuteri* 100-23 Δ*gls*1-2-3 and wild-type strain were further explored in sourdough fermentation. Whole wheat flour sourdough were prepared with each strain and incubated at 37°C for 96 hours. Figure 5 demonstrated the concentration of the three metabolites quantified by HPLC.

The concentration change of the three compounds in figure 5, clearly showed that *L. reuteri* 100-23 contains the ability of converting glutamine to glutamate then to GABA. The accumulation of GABA reached 57.7 ± 6.0 mmol/kg of sourdough after 96 hours of fermentation; but the concentration of the other two metabolites remained below 20 mmol/kg, indicating that the wild-type fermented sourdough does not accumulate glutamine or glutamate. In addition, *L. reuteri* 100-23 Δ*gls* 2-3 was added as a comparison and it behaved very similar to that of the wild-type throughout fermentation.

L. reuteri 100-23 Δ*gad*B accumulates glutamate (Su et al., 2011). The concentration of glutamate increased gradually during 96 hours of incubation and yielded to 65.2 ± 0.3 mmol/kg at the end of fermentation as shown in figure 5. Besides, this mutant lost the ability to produce GABA, as the concentration of GABA remained insignificant comparing to the other strains tested (lower or equal to the chemically acidified treatment).

Glutamine accumulation in *L. reuteri* 100-23 Δ*gls*1-2-3 is clearly showed in figure 5. The strain lost *gls*-related activities, as the three glutaminases coding genes were knocked out. However, the mutant showed comparable levels of glutamate to that of the chemically acidified group. Figure 5 listed that the concentrations were 42.1 ± 5.6 mmol/ kg of glutamine and 45.7 ± 1.2 mmol/kg of glutamate after 96h fermentation, respectively. GABA levels was relatively high for the triple knock-out mutant, which was in accordance with the GABA content of pH 6.2 buffer assessment observation.

The changes of glutamine and glutamate levels in sourdough over 96 hours fermentation indicated that glutamine concentration of *L. reuteri* 100-23 and *L. reuteri* 100-23 Δ*gad*B remained considerably low comparing to the chemically acidified treatment. Taken the cell count

and pH into consideration as listed in figure 4, glutamine deamidation is mainly used for growth for the first 24 hours; and after the first 24 hours of propagation, as the pH dropped to 3.5, glutamine is utilized for the purpose of acid resistance. *L. reuteri* 100-23 Δ*gad*B, in which the conversion of glutamate is disrupted, distinctly showed the gradual accumulation of glutamate over time in figure 5. The content of the three metabolites throughout fermentation is a straightforward demonstration that glutaminase activity is the first step of glutamine metabolism under acidic condition, where glutamine is converted to glutamate by *gls* activity and therefore protects the cells from acid stress.

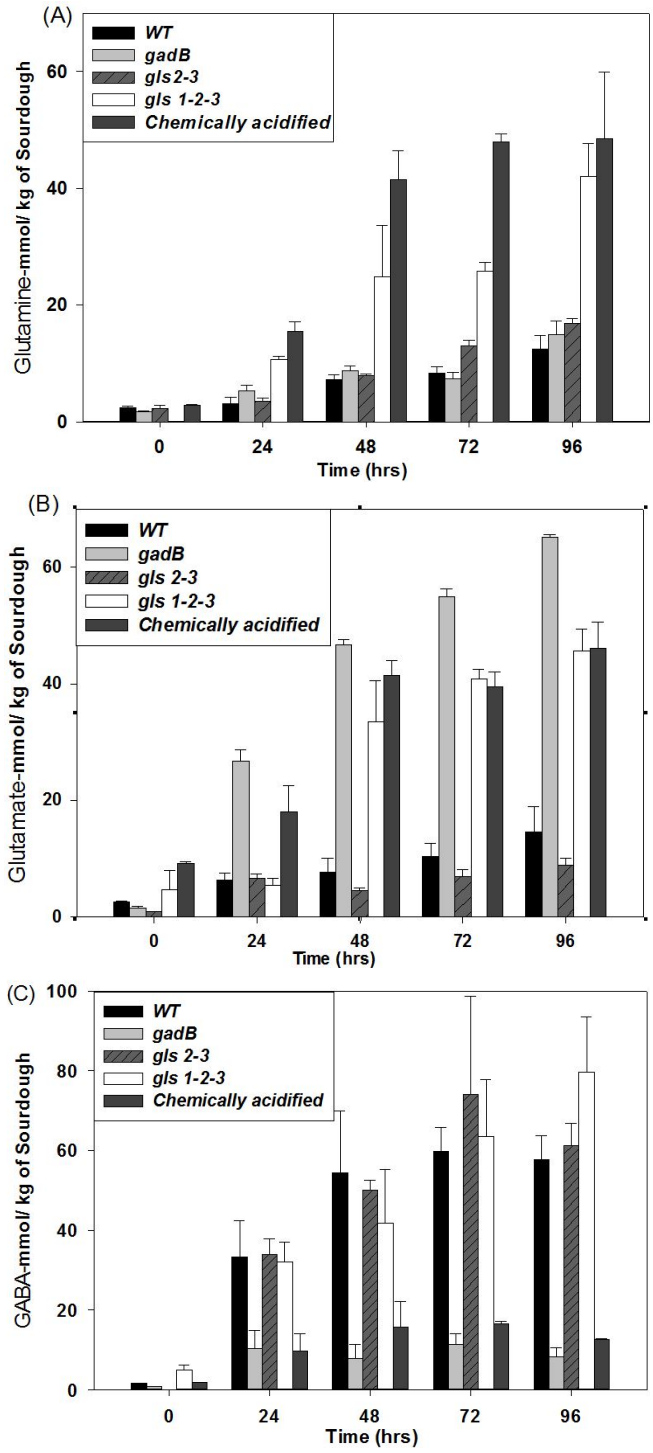

Figure 6. Concentration of glutamine **(A)**, glutamate **(B)** and GABA **(C)** during sourdough fermentation at 37°C over 96 h with *L. reuteri* 100-23 (**WT**), *L. reuteri* 100-23 Δ*gad*B (**gadB**), *L. reuteri* 100-23 Δ*gls* 2-3 (**gls2-3**), *or L. reuteri* 100-23 Δ*gls*1-2-3 (**gls1-2-3**), chemically acidified sourdough (**Chemically acidified**). Columns represent means ± standard deviation from independent triplicate fermentations.

3.5 Glutamine, glutamate and GABA in sourdough bread

Whole-wheat sourdough breads were prepared with 6% sourdough incorporated. Bread samples were freeze dried, and concentrations of glutamine, glutamate and GABA were also evaluated. Results were plotted in figure 6.

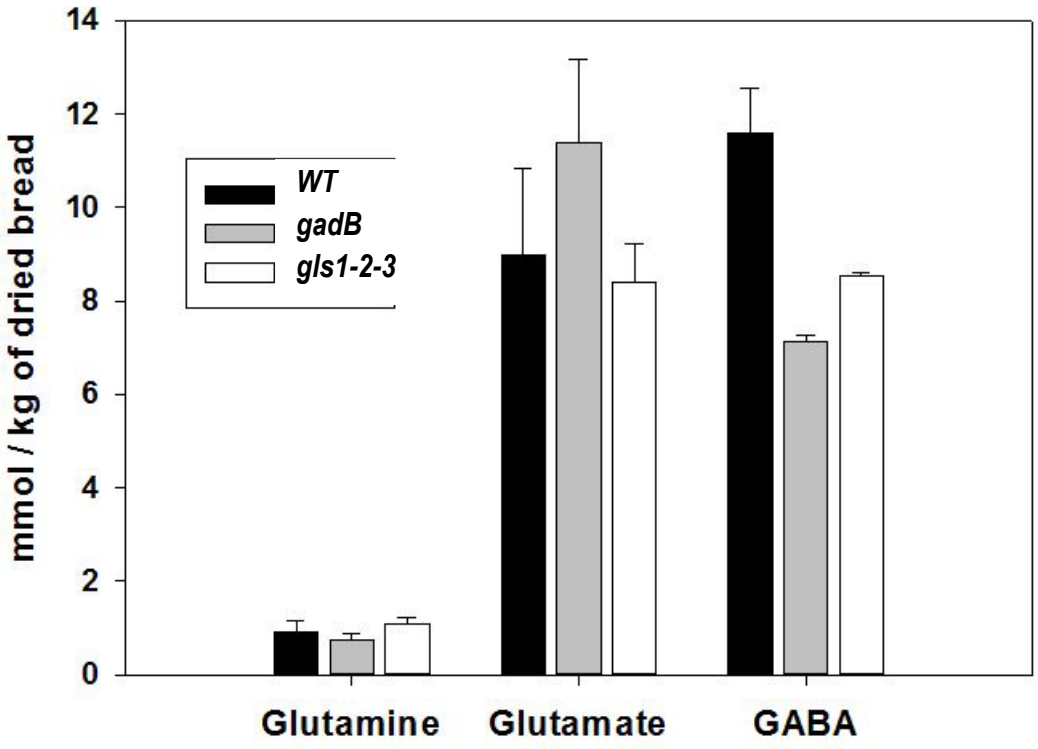

Figure 7. Concentration of **Glutamine**, **Glutamate** and γ-aminobutyric acid (**GABA**) of Freeze-dried bread: produced by 6% sourdough inoculum, fermented with *L. reuteri* 100-23 (**Black bar**), or *L. reuteri* 100-23 Δ*gad*B (**Grey bar**), *or L. reuteri* 100-23 Δ*gls*1-2-3 (**White bar**). Columns represent means ± standard deviation from three repeats.

Figure 6 demonstrated the change in terms of concentration for glutamine after baking. After the heat treatment, the data showed that *L. reuteri* 100-23 Δ*gls* 1-2-3 fermented sourdough bread still contained the most glutamine among the three breads, with 1.1 ± 0.1 mmol glutamine per kg of dried bread. There is a difference tendency towards the level of glutamine between *L. reuteri*

100-23 Δ*gls* 1-2-3 sourdough bread and *L. reuteri* 100-23 Δ*gad*B produced sourdough bread, where the P value of 0.0603 is less than 0.1 yet larger than 0.05.

Glutamate content remained the high in bread produced with *L. reuteri* 100-23 Δ*gad*B, which is explainable since the ability to metabolize glutamate was disrupted by the disruption of glutamate decarboxylase.

Sourdough bread produced with *L. reuteri* 100-23 had the highest level of GABA, 11.6 ± 1.0 mmol/ kg of dried bread. Moreover, GABA content suggests a significant difference between *L. reuteri* 100-23 sourdough bread and *L. reuteri* 100-23 Δ*gad*B bread, for which the P value was 0.0065.

3.6 Sensory evaluation of sourdough bread

The most abundant amino acid in wheat proteins is glutamine. Glutamine is converted to glutamate or γ-aminobutyrate by glutaminase in *Lactobacillus reuteri* and other sourdough lactobacilli. Sensory evaluation was conducted to understand the consumers' perspective of taste on bread produced with different *L. reuteri* strains.

3.6.1 Demographic information

Forty-one recruited panelists successfully completed the sensory evaluation at University of Alberta AFNS, in a sensory laboratory with controlled white lighting, 22°C, 45% RH and positive pressure. Majority of the participants were female and 71% of the people belong to the age group of 18-29 years old. 88% of the panellists were either familiar with or heard of sourdough bread.

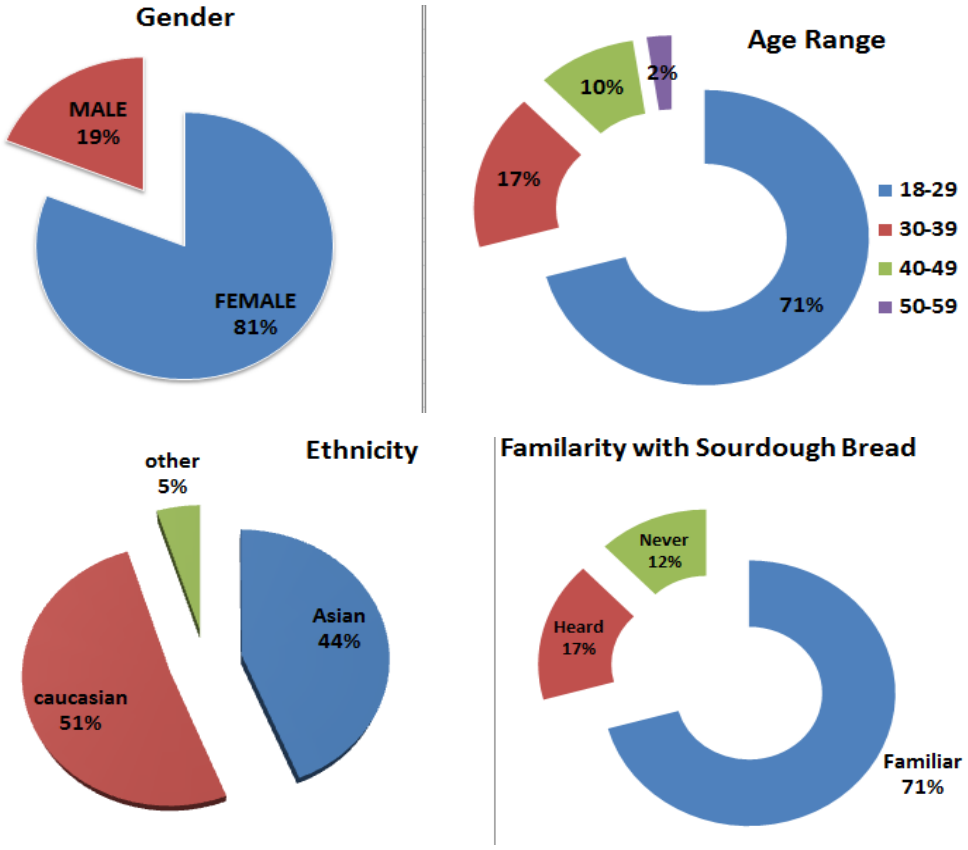

Figure 8. Demographic summary of the consumers participated in the sourdough bread sensory evaluation, n=41.

3.6.2 Triangle tests

The impact on the taste of sourdough bread depends on the metabolism of glutamine during fermentation. Glutamine is tasteless, while glutamate is the major contributor to savory "umami" taste.

Two sets of triangle test were implemented at the first part of the sensory evaluation, to investigate whether or not the consumers could differentiate the sourdough bread fermented with various strains of *L. reuteri* 100-23.

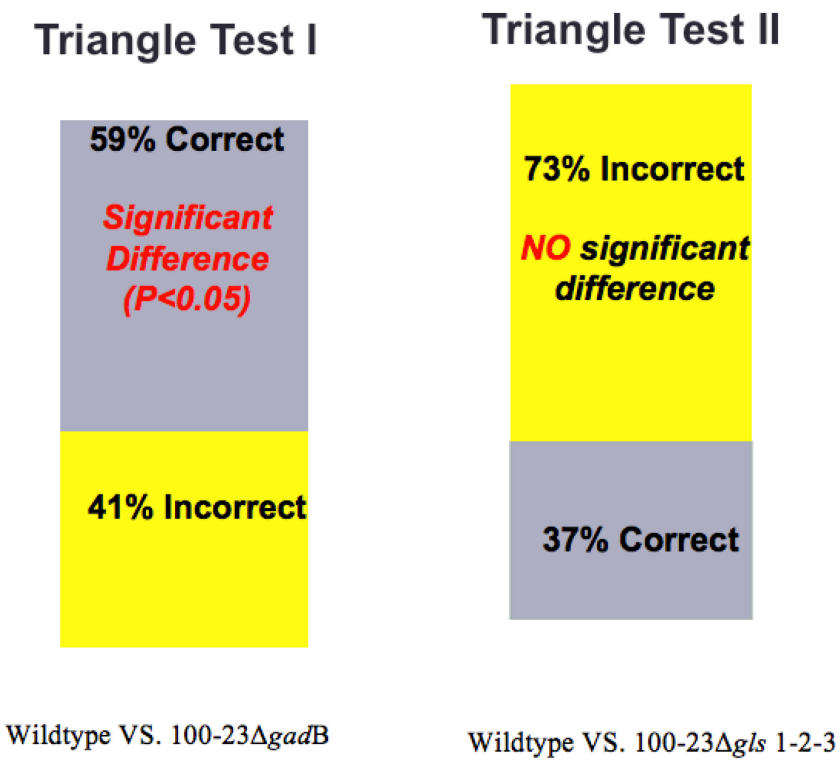

Figure 9. Differentiation of breads by Triangle test. Identification in terms of percentage: the results from 41 participants who identified the odd sourdough sample in triangle tests with random codes. Significance is determined according to the minimum numbers of correct judgments to establish significance in a triangle test (Lawless & Heymann, 2010).

Evaluation of sourdough bread fermented with *L. reuteri* strains demonstrated a significant difference between the glutamate accumulating *L. reuteri* 100-23 Δ*gad*B bread and the GABA (γ-aminobutyrate) accumulating wild type *L. reuteri* 100-23 bread. Between the two breads, 24 participants correctly identified the odd sample among the three, which exceeds the minimum number of correct response required (19, $p< 0.05$) according to the minimum numbers of correct judgments to establish significance in a triangle test (Lawless & Heymann, 2010).

In contrast, bread produced with the *L. reuteri* 100-23 Δ*gls*1-2-3, which does not convert glutamine to either glutamate or γ-aminobutyrate, was not different from bread produced with *L. reuteri* 100-23. Only 15 accurate identifications were collected out of the 41 panellists.

3.6.3 Attributes ranking test

Friedman rank test (Lawless & Heymann, 2010) was employed (score 1-9), to better understand consumers' perception of the sourdough bread in terms of saltiness, sourness, overall taste and overall preference.

As shown in Table 4, bread fermented with the *L. reuteri* 100-23 Δ*gls*1-2-3 was rated 6.244 and 6.195 out of 9.0 on overall taste and likeness; while the wide type produced bread scored 5.976 and 5.878 for overall taste and likeness, respectively.

ANOVA tests indicated that none of the four attributes assessed were rated significantly different among the three sourdough breads ($p < 0.05$).

The difference detected in the triangle test between the glutamate accumulating *L. reuteri* 100-23 Δ*gad*B bread and the GABA (γ-aminobutyrate) accumulating wild type *L. reuteri* 100-23 bread was not derivable to the specific attributes assessed.

TABLE 3. ANOVA for Attributes ranking of the sourdough bread evaluation

(n=41, score range 1-9)

Attributes	Treatments			Significant Difference (p <0.05)
	100-23	100-23 Δ*gad*B	100-23 Δ*gls*1-2-3	
Saltiness	4.439	4.512	4.561	NO
Sourness	4.536	4.195	4.658	NO
Overall Taste	5.976	6.146	6.244	NO
Overall Likeness	5.878	6.049	6.195	NO

4 DISCUSSION

This project was designed to investigate the role of glutaminase activity in *L. reuteri* 100-23, in terms of their contribution to acid resistance and glutamine metabolism throughout sourdough fermentation.

4.1 Double crossover mutagenesis

Disruption or interruption of a targeted gene is usually achieved through homologous recombination (Walter et al., 2005), which involves integrating a shuttle plasmid in to the host. However, the insertion mutation can be problematic considering the stability of the shuttle plasmid, as well as the requirement of antibiotics supplementation. Antibiotic resistance is a major ethical concern for sensory evaluation.

Recombineering is an efficient homologous recombination method *in vivo*, which allows precise construction of DNA molecules junctions and avoids the use of restriction enzymatic sites (Sharan et al., 2009). Single-stranded DNA (ssDNA) recombineering has been applied in *Lactobacillus reuteri* by combining CRISPR-Cas9 (van-Pijkeren et al., 2012).

Double crossover mutagenesis is an alternative method to generate a deletion mutant. In this project, multiple deletions are required since *L. reuteri* 100-23 contains three coding genes for glutaminases (Teixeira et al., 2014). With the availability of the single-deletion mutant *L. reuteri* 100-23 Δ*gls*1, *L. reuteri* 100-23 Δ*gls*2 and *L. reuteri* 100-23 Δ*gls*3, as well as the plasmids with *gls* region disrupted, the double crossover mutagenesis method is desirable. This arrangement eliminates the concerns of shuttle plasmid transposition or interference of foreign genes; moreover, the deletion mutant is antibiotic-resistance free and the applications can be extended comparing to an insertion mutant (Su, 2011). The in-frame deletion minimizes the polar effects of the down-stream gene expression. Therefore, sourdough bread fermented with the antibiotic-resistance free mutant is suitable for sensory evaluation.

It is necessary to point out that this *in-cis* replacement of mutation is not perfect. The construction of the mutant involves ligation of the upper and down stream of the targeted gene,

which means that the adjacent region of the targeted coding piece might be affected or altered through the process.

4.2 Acid resistance of *L. reuteri* 100-23

4.2.1 Overview of glutamine-mediated acid tolerance in *L. reuteri* 100-23

Glutamine is converted to glutamate by glutaminases and produces ammonia at the same time. The free ammonia neutralizes proton, therefore regulate the pH when encounters acid pressure (Lu et al., 2013).

Glutaminase's association with acid resistance was first thoroughly established and characterized in *E. coli*; Lu and others reported the identification of a two-component acid resistance system that is comprised of the glutaminase YbaS and the amino acid antiporter GadC in *E. coli*. The optimal pH for YbaS and GadC is 6.0 and below and they are activated by acidic pH, which is in accordance with their acid resistance responsibility (Lu et al., 2013).

Glutaminase activity is strain specific in lactic acid bacteria and *L. reuteri* 100-23 harbors three functional glutaminases: *gls*1, *gls*2, and *gls*3 (Teixeira et al., 2014). Since glutamine is the most abundant amino acid in wheat proteins and proteolysis of wheat proteins releases high levels of glutamine (Gänzle et al., 2007), the glutaminase acid-resistance system identified in *E. coli* is likely to have a key role for the survival in *L. reuteri* 100-23. Relevant studies showed that *Lactobacillus sanfranciscensis* had a higher final pH in the 75mmol glutamine-containing medium than the final pH in regular mMRS medium (Vermeulen et al., 2007); and Lackovic et al. (2001) reported that comparing to the *Streptococcus mutans* wild type, strains without the glutamine transport system was more sensitive against acid stress; both suggesting the association between glutaminase/ glutamine antiport and acid resistance.

The function of glutaminase in acid-tolerant *Lactobacillus* is not fully understood, hence, this study aimed to understand whether glutamine conversion to glutamate by glutaminase activity improves the survival of *L. reuteri* under acidic conditions independent of other amino acids conversion. In terms of gene expression among the three glutaminases, all expressed during growth; in particular, *gls*3 and *gad*B were correspondingly over-expressed, which is in accordance with the sequential conversion between glutamine, glutamate and GABA in *L. reuteri* 100-23 (Teixeira et al., 2014).

Two pH levels were selected in this study: a pH of 2.5 adjusted with HCl was chosen to match the gastric fluid environment and a pH of 3.5 was selected to mimic the sourdough fermentation condition and the fore-stomach environment. Glutamine and glutamate metabolism implies a role of glutamine to acid resistance in *L. reuteri* 100-23 (Vermeulen et al., 2007). Glutamate supplementation helped the survival of *L. reuteri* 100-23 at pH 2.5 (Su et al., 2011; Teixeira et al., 2014), while this study suggests the protective effect of glutamate on *L. reuteri* 100-23 at pH 3.5.

Glutamine supplementation significantly increased the survival of *L. reuteri* 100-23 Δ*gad*B at pH 2.5 (Teixeira et al., 2014); it was confirmed and extended in this project at both pH 2.5 and 3.5. This study determines that glutamine deamination under acidic environment is beneficial to *L. reuteri* 100-23 survival. The survival of *E. coli* at pH 2.5 was improved significantly with the supplementation of glutamate and glutamine but not with arginine or other amino acids; and GadC has been pinpointed to transport glutamine in *E. coli* (Lu et al., 2013), while in *L. reuteri* 100-23 the antiporters of glutamine remains to be identified. GadC is a membrane transporter that transports GABA/Glutamate and it is functional only at pH lower than 6.5 (Ma et al., 2012). Regardless of the impaired Glutamate/GABA antiport under acidic conditions in minimal media, intracellular GABA accumulation is observed in *Listeria monocytogenes* cells; intracellular GAD system is the more active towards acid pressure (pH 4.0-5.0) than the extracellular one (Karatzas et al., 2012).

Foster (et al., 2004) pointed out that amino acid-dependent acid resistance systems contribute to converting inside negative membrane potential to positive charge. Thus, reversing trans-membrane potential and maintaining a stable intracellular pH value consists the acid resistance strategy in *E. coli*.

Intracellular pH plays a crucial role in the transportation of glutamine/glutamate. The selection of ions being transported determines the neutralization of protons and the polarization of the membrane; when pH is lower than 4.0, the γ-carboxyl group (pKa 4.25) is mostly protonated, therefore neutral charged glutamate is the prevailing form (Teixeira et al., 2014); membrane polarization and cytoplasmic pH escalation was observed in *L. reuteri* through the action of glutamate decarboxylase (Teixeira et al., 2014; Gänzle, 2015).

4.2.2 Acid resistance of *L. reuteri* 100-23 Δ*gls*1-2-3

Cell counts indicated that pH=2.5, additional glutamine or glutamate does not aid the survival of *L. reuteri* 100-23 Δ*gls*1-2-3 (Figure 2). Without the glutaminase activity, it is understandable that glutamine supplementation is no longer protective for the cell; however, glutamate addition benefits the survival of wild-type but not the glutaminases deletion mutant. The survival pattern continued when the pH was adjusted to 3.5, in which glutamate addition was the least favorable to acid stress in pH=3.5 phosphate buffer for *L. reuteri* 100-23 Δ*gls*1-2-3 (Figure 3).

The glutamate transporter, aside from protonated glutamate, can also transport glutamine with a high affinity (Oppedisano et al., 2007). The competition over anti-porters and pH limitation could be the reasons why *L. reuteri* 100-23 Δ*gls*1-2-3 does not profit from glutamate supplementation. Moreover, with the disruption of the glutamine deamidation pathways, the mutant could rely on other outlets when encountered with acid stress.

Lactic acid bacteria transportation of glutamine and glutamate has been reported for *Lactobacillus casei* (Strobel et al. 1989). GlnPQ, an ABC transporter in *Lactococcus lactis*, transport glutamine / glutamate but inhibited by arginine and γ-glutamylhydrazide; and the transporter activity is associated with internal pH, for which the glutamate uptake peaks at internal pH ~7.3 while inactive below pH 6 (Schuurman-Wolters & Poolman 2005). The pH dependence of GlnPQ suggests that the transporter is relevant to cell growth and anabolism but not to acid resistance.

In *L. reuteri* 100-23, it has been demonstrated that the conversion of glutamate to GABA by glutamate decarboxylase contributes to acid resistance (Su et al., 2011); In *E. coli,* an amino acid antiporter GadC that exchanges extracellular glutamine or glutamate with intracellular GABA, and GadA and GadB- two glutamate decarboxylases that catalyze the reaction of glutamate to GABA, take part in the acid resistance system (Foster, 2004; Lu et al., 2013).

Another acid resistance system for pH homeostasis is associated with arginine metabolism. In lactic acid bacteria, it is achieved through the arginine deiminase (ADI) pathway, in which the acidic pressure is relieved by intracellular consumption of protons and the production of ATP (Cunin et al. 1986; Konings, 2002). Arginine deiminase activity contributes to the acid resistance of *L. reuteri* CRL 1098 (Rollan et al. 2003), but it is absent in *L. sanfranciscen*sis DSM20451

(Vermeulen et al., 2006). Survival of *L. reuteri* at pH 3.5 were improved with arginine supplementation and *L. reuteri* was enhanced by additional glutamine or glutamate at pH 2.5, indicating that acid resistance mechanisms are supplementary to each other while under different conditions (Teixeira et al., 2014).

In addition to glutamine / glutamate or arginine based acid tolerance, amino acids based pH homeostasis of lactic acid bacteria also involves the decarboxylation of histidine, ornithine or tyrosine (Gänzle, 2015). *L. reuteri* possesses histidine decarboxylase (Hdc) while ornithine decarboxylase (OrnDC) and tyrosine decarboxylase (TyrDC) are absent (Zheng et al., 2015a). Urease production in rodent isolate *L. reuteri* 100-23 is associated with acid tolerance (Wilson et al., 2014); however, urea hydrolysis is irrelevant in food fermentation (Zheng et al., 2015a).

4.3 Effect of glutamate producing *L. reuteri* on the taste of bread

Disruption of the glutaminases leaves an impact on the accumulation of the taste active glutamate, therefore, sensory evaluation of sourdough breads produced with various *L. reuteri* was conducted to determine the influence of the glutamine metabolism on bread taste. 6% of sourdough inoculum was chosen for the bread sensory evaluation after pre-test of three various inoculum levels, 3%, 6% and 10%. It is difficult to detect the differences between the 3% sourdough breads and the 10% inoculum is unacceptable for consumer panel, in which the pungent acidic flavour overshadows any other odor or taste.

Glutamate is the major contributor to the umami flavor ("savory taste"); whereas glutamine is a tasteless amino acid that is frequently associated with bodybuilding supplements (Colker et al., 2000). Sourdough bread showed improved flavor with additional amino acids to the dough as well as with the addition of fungal protease (Thiele et al., 2002). It is suggested that glutamate formation, aside from the formation of volatiles from amino acids during baking, may contribute to bread flavor (Schieberle, 1996). Lactobacilli convert glutamine into glutamate during growth in sourdough and glutamate production in sourdough became a factor to prompt the flavor of the bread (Vermeulen et al., 2007).

Considering the HPLC analysis of amino acids concentration in both sourdough and sourdough bread samples, together with the sensory evaluation results, it can be concluded that sourdough bread fermented with *L. reuteri* strains demonstrated a significant difference in terms of taste

between the glutamate accumulating *L. reuteri* 100-23Δ*gad*B and the γ-aminobutyrate accumulating wild type *L. reuteri* 100-23, according to the sensory evaluation of 44 untrained panelists. The taste threshold of MSG in bread is approximately 2 mmol/kg (Zhao et al., 2015). In this study, the glutamate level in bread produced with *L. reuteri* 100-23, *L. reuteri* 100-23Δ*gadB* and *L. reuteri 100-23* Δ*gls*1-2-3 was 8~12 mmol/kg, which is 3~5 folds higher than the threshold. This correlates with the previous study where a consumer panel detected significant difference between glutamate- and GABA- producing *L. reuteri* sourdough bread; and a trained panel identified the specific feature to be "umami" (Zhao et al., 2015). As listed in Table 4., the untrained participants did not pinpoint the taste difference onto specific attribute assessed.

On the other hand, bread produced with the *L. reuteri* 100-23Δ*gls*1-2-3, which does not convert glutamine to either glutamate or γ-aminobutyrate, was not different from bread produced with *L. reuteri* 100-23. This can be explained by the glutamate levels during sourdough fermentation, in which the concentration was identical for both of the chemical acidified control and the *L. reuteri* 100-23Δ*gls*1-2-3 treatment (Figure 5), suggesting chemical deamidation of glutamine during type II sourdough fermentation.

4.4 Glutamine and Glutamate metabolism in *L. reuteri* 100-23

The glutamine and glutamate metabolism in *L. reuteri* 100-23 is not simply mediated by the two enzymes glutaminase and glutamate decarboxylase, based on the metabolites levels monitored throughout the sourdough fermentation (Figure 5). Functional genes screening through the database of IMG (Integrated Microbial Genomes) shows that *L. reuteri 100-23* harbors one coding region for glutamine synthetase- glnA (GS, EC. 6.3.1.2).

Glutamine synthetase (GS) is an enzyme that catalyzes the condensation of glutamate and ammonia and produces glutamine: glutamine generation is favored when an ammonium ion attacks the acyl-phosphate, and if water attacks the intermediate then glutamate production is preferred (Eisenberg et al., 2000). In *Lactobacillus sanfranciscensis*, glutamine deamidation by crude cell extracts was detected, indicating glutaminase activity rather than glutamine synthetase activity (Vermeulen et al., 2007).

During sourdough fermentation, cells and metabolites were exposed to acidic environment (pH 3.5-4.3) for days (Thiele et al, 2002), this condition could affect the chemically deamidation of

glutamine. A more facile deamidation of glutamine residue in glucagon fragment 22-29 when encounters acidic conditions was observed; degradation at pH 2.5-3.0, 60°C for 70 h showed that glutaminyl deamidation at glucagon fragment 3, 20, and 24 (Joshi et al., 2000; Joshi et al., 2002). Glutamine is converted to cyclic 5-oxoproline in water at neutral pH when heated to 100°C, and furthermore, the production of glutamate occurs when dilute aqueous HCl is presented, because cyclic 5-oxoproline could react with H_2O (Halim et al., 2014). This could explain that glutamate accumulation was not solely by the action of glutaminases, but the combination of the alternative metabolic and degradation pathways.

The keto-acid product of glutamate deamination, α -ketoglutarate (α -KG), also takes part in the metabolism of glutamine and glutamate in *L. reuteri* 100-23. In *Lactobacillus lactis* and certain lactobacilli, it was shown that α -KG was produced from glutamate via the action of a glutamate dehydrogenase. A study showed the production of α-KG via the action of citrate permease, citrate lyase and aspartate aminotransferase was found to be functional in *Lactococcus lactis* subsp *diacetylactis* (Tanous et al., 2005). *Lactobacillus sanfranciscensis* DSM20451 demonstrated glutamate dehydrogenase activity, converting glutamate to α-ketoglutarate (Vermeulen et al., 2006). In bacteria, thus conversion can go both ways, depending on the environment. It is a crucial intermediate in Kerbs cycle and formation of other amino acids (Tanous et al., 2005). Screening through RAST database (rapid annotation using subsystem technology, V2.0) indicates that *L. reuteri* does not contain the full citric acid cycles.

Taken together, a proposed glutamine and glutamate metabolic pathways in *L. reuteri* 100-23 was summarized Figure 9. Glutamine deamidation during sourdough fermentation could be a competitive advantage for lactic acid bacteria growth and survival; ammonia is preferred as the nitrogen source over amino acids for yeast (Gobbetti, 1998; Vermeulen et al., 2007). Glutamine and glutamate metabolism in *L. reuteri* contributes to acid resistance and leaves an impact on the flavor and taste of bread (Su et al., 2011; Teixeira et al., 2014).

5 CONCLUSIONS AND FUTURE PERSPECTIVES

This study aimed to understand the role of glutaminase activity in *L. reuteri* 100-23.

In terms of contribution to acid resistance, glutaminase activity enhances the survival of *L. reuteri* 100-23 in both acidic environments (*in vitro*) and sourdough fermentations (*in vivo*) by deamidation of glutamine to glutamate. Analysis of the glutamine / glutamate dependent acid resistance demonstrated that glutamine deamidation improved acid resistance independent of glutamate decarboxylation. By disruption of the three glutaminase genes, cells were still resistant under acid pressure, indicating that *L. reuteri* 100-23 acid resistance does not solely rely on glutaminase activity. Literature review suggests that other mechanisms are involved in *L. reuteri* acid resistance, including antiporters of glutamine (Lu et al., 2013); arginine deiminase (ADI) pathway (Rollan et al. 2003), glutamate decarboxylase activity (Su et al., 2011) and histidine decarboxylase (Gänzle 2015; Zheng et al., 2015a).

For *L. reuteri* 100-23 glutamine metabolism, alternative way of deamination (i.e. chemical deamidation) that accumulates glutamate was observed during long-hour sourdough fermentation (37°C, 96h). Evaluation of sourdough bread fermented with *L. reuteri* strains demonstrated a significant difference between the glutamate accumulating *L. reuteri* 100-23Δ*gadB* and the GABA accumulating wild type *L. reuteri* 100-23. In contrast, bread produced with *L. reuteri* 100-23 Δ*gls*1-2-3, which does not convert glutamine to either glutamate or γ-aminobutyrate, was not different from bread produced with *L. reuteri* 100-23.

Glutamine conversion of sourdough lactobacilli contributes to acid resistance, and enhances the taste of bread. A better understanding of *L. reuteri* to cereal fermentation ecosystems is achieved through the extended information on acid resistance and glutamine conversion of the *L. reuteri*, assisting the selection of strains that can be used as starter cultures for baking improvers (i.e. production of salt reduced bread).

Exploration on the changes of transmembrane electrical potential and the intracellular pH measurements under acidic condition would further facilitate the understanding and characterization of glutaminase activity to acid resistance.

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APPENDICES

I. Project Information Sheet: Consumer Panel Sensory Evaluation of Sourdough Bread

Purpose: The purpose of this project is to evaluate consumer perception and preference for sourdough bread.

Consumer panel Methods: You are being asked to voluntarily participate in a consumer sensory panel to taste three samples of sourdough bread and to evaluate each sample in terms of two sensory attributes and tell us which product you prefer. The products have been prepared in AFNS food laboratory. You will also be asked to complete a questionnaire about your bread consumption habits and provide some demographic information such as your gender and age range. The session will take about 10 minutes.

Confidentiality: You are not asked to provide your name on any of the questionnaires; participation is anonymous. Your questionnaires will be given a participant number. Only the student research team will have access to your data.

Benefits: The results of this study may not have any direct benefits for you. However, the practice of sensory panel management and results from this study will be valuable to the graduate students who are completing this sensory project as part of the research.

Risks: The risks of participating are no different from the normal risks associated with the consumption of wheat bread. The ingredients are as follows:

<u>Sourdough bread</u>

- Water
- All-purpose wheat flour
- Dried sourdough
- Sugar
- Salt

- Yeast

If you have allergies, intolerances, or sensitivities to any of these ingredients you should not participate.

Withdrawal from the Study: Even after you have agreed to participate in the consumer panel, you may withdraw from the session at any time before or during the evaluations. The students will not use any information you have given to that point.

Use of Your Information: This study is being done by graduate students in the Department of Agricultural, Food and Nutritional Science for partial fulfillment of thesis research requirement. Your data will be combined with data from other participants and will be used for a project paper and oral presentation describing the research study and results.

For further information about the study, you can contact:

Qianying Tao, qt1@ualberta.ca or Dr. Michael Gaenzle, mgaenzle@ualberta.ca

For information about how this project is carried out you may contact:

The Research Ethics Office at 780-492-2615. This office has no affiliation with the study investigators.

The plan of the study has been reviewed for its adherence to ethical guidelines and approved by the Research Ethics Board at the University of Alberta.

II. Consent Form: Consumer Panel Sensory Evaluation of Bread

Please circle your answers:

Do you have any allergies, sensitivities or intolerances to any of the following ingredients?	Yes	No
Sourdough bread • Water • All-purpose wheat flour • Dried sourdough (wheat) • Sugar • Salt • Yeast		

If you have answered" yes", please stop and tell us immediately.

Do you understand that you have been asked to be in a student project research study?	Yes	No
Have you read and received a copy of the attached Information Sheet?	Yes	No
Do you understand the benefits and risks involved in taking part in this research study?	Yes	No
Have you had an opportunity to ask questions and discuss this study?	Yes	No

Do you understand that you can quit taking part in this study at any time?	Yes	No
Has confidentiality been explained to you?	Yes	No
Do you understand who has access to your data?	Yes	No
Do you know what the information you say will be used for?	Yes	No
Do you give us permission to use your data for the purposes specified?	Yes	No

This study was explained to me by

I agree to take part in this study.

_____	_____	_____/_____/ 2015
Signature of Research Participant	Printed Name	Date (dd/mm/yyyy)

I believe that the person signing this form understands what is involved in the study and voluntarily agrees to participate.

Signature of Investigator or Designee

III. Sourdough Bread Sensory Evaluation (Demographic) Participant # _____

1. Please indicate your gender: ☐ Male ☐ Female

2. Please indicate the age group that you belong to:

☐ 18 years below ☐18-29 years ☐ 30-39 years

☐ 40-49 years ☐50-59 years ☐ 60 years and plus

3. Which of the following breads would you consider to be your favourite?
☐ Whole Grain Bread ☐ White Bread ☐ Brown Bread ☐ Flat Bread

4. On average, how often do you consume bread?
☐ Once a day ☐ 2 – 5 times a week ☐ Once a week

☐ Every 2 -3 weeks ☐Never

5. Are you familiar with sourdough bread?
☐ Yes ☐Heard of it, but never tried it before ☐Never heard of it

6. What is your ethnicity?
☐ Caucasian ☐ African-American ☐ Asian ☐ Other

7. Please indicate your highest education level achieved
☐ Less than high school ☐ High school ☐ 2-year College degree

☐ 4-year College degree (BA, BS) ☐ Master's degree ☐ Doctoral degree

Thank you for taking the time to fill out our survey 

IV. Bread Baking Protocol

1. **Turn on the proof chamber** ----2 buttons (time and temperature)
   Add 2 cups (medium yogurt cup) of water;
   Keep at **30 C;**

2. **Activate yeast by mixing**
   2g yeast
   2g sugar → put into proof chamber → @30-40 C for 15 mins
   65mL H2O

3. **Weigh**
   1) 94g All-purpose wheat flour
   2) 2g Table salt-precisely!!!
   3) 6g Freeze-dried sourdough flour (6%-based on 100g of flour)

4. **Kneading----Kitchen Aid**
   1) Right side---lock
   2) Left side----- @ Speed 2—2 mins
      @ Speed 5~6—3 mins

5. **Grease the baking tray; Transfer the dough to proof chamber @ 30C for 1-1.5 hours Check volume**

6. **Dough shaping**

7. **Resting @ 30C for 1-1.5 hours; Check volume**

8. **Baking @ 210C(350F) for 25 mins**

9. **Cooling @room temperature for 2-3 hours.**

VI. Plasmids Construction Information (Su, unpublished)

Gene information	Primers (5' to 3')

gls1

> In Marcia and Sabine's lab books, it is 69653 or 653 referred to 2500069653 L-glutaminase;. For publications, it should be *gls1*

> 921 bp

ATGAATCTTAATGACGCAATTCAAGAATGT
TGGAGCAAAATTGACGAGGGACAGGTAGCA
ACCTATATTCCTGCTTTAGCAAAAGTTGAC
CCTTATCAACTTGGCGTTTACCTTTTTGAC
GTCACAAATGATAAAAAAGTAGAAGCAGGC
GCATCGCAAGTTCGGTTTGCTATCGAAAGT
GTCTCGAAAGTAATCACCCTCCTCTACGCA
ATTGAACGTTTAGGCTTGTCAGCAGTTGAA
GAGCAAGTCGGAACACGCCAGACCGGCTTC
CCGTTCGATACAATCCTTAACATGGAAATC
ACTAAAGAGACCCACCCTCTCAATGCTTTT
ATTAATAGTGGTGCCATCCTCATCAGTTCA
TTAATCGAAGAACAAGATGGGCTTTCTCCC
TTTGACCAAATCCTTGAATTTAGTCGTAAA
ATTTGCAATGATCCTGATATCACCCTCAAT
GAAGAAATTTACCAATCGGAGTTGCGAACC
GGGGATATGAATCGGTCGTTAGCCTACTAT
CTCAAGGCCAAAGAAGTCCTCACTAATGAT
GTAACCCTTAGCCTTGATACCTATTTTAAG
CAGTGTTCAATGATGGTCACTTGCCAAAGT
CTTGCTAACCTCGGTGCTGTCCTTGCCAAT
GACGGGATCGCTCCTTGGAATAATGAACGA
ATCATTTCAAGCGAGGCTGCTACATATACA
AAATCGGTCATGATGACGACTGGCCTCTAC
AATGAATCAGGAACTTATTCCGTTCGAATC
GGTGTACCGACTAAGAGCGGTGTCGGTGGC
GGTTTAGTTTCTGCTGCCCCCAATCATTAC
GGGATCGGAATTTTCAGTCCGGCACTCGAC
CATGCTGGTAATAGTGTTGCCGGGCTAGCA
CTCCTTGAACTCATCAGTAAAAAATTAAAG
CTTGATATTTTTAGGTACTAG

E. coli JM109 pJRS pKO-*gls*1-AB Primers

gls1-KO1-PstI

AACTGCAGGGGATTGTAACTTGAAATTAAC

gls1-KO2-BglII

GAAGATCTTCATTCTTGAATTGCGTCATTAA G

gls1-KO3-BglII

GAAGATCTAGGTACTAGTTGCAAATATTCGC

gls1-KO4-BamHI

CGGGATCCGATATTCAGCAGTCGAAAG

Amino acid sequence Gls 1; 306 aa

MNLNDAIQECWSKIDEGQVATYIPALAKVDPYQLGVYLFDVTN
DKKVEAGASQVRFAIESVSKVITLLYAIERLGLSAVEEQVGTR
QTGFPFDTILNMEITKETHPLNAFINSGAILISSLIEEQDGLS
PFDQILEFSRKICNDPDITLNEEIYQSELRTGDMNRSLAYYLK
AKEVLTNDVTLSLDTYFKQCSMMVTCQSLANLGAVLANDGIAP
WNNERIISSEAATYTKSVMMTTGLYNESGTYSVRI
GVPTKSGVGGGLVSAAPNHYGIGIFSPALDHAGNSVAGLALLE
LISKKLKLDIFRY

gls2

> In Marcia and Sabine's lab books, it is 70771 or 771 referred to 2500070771 L-glutaminase;. For publications, it should be *gls2*

>915 bp

ATGCCTTGCGTGAGAGAGGTTTTGACGATG
CAAAAATTAGAGCAATTGATTGATAAAAAC
TTTGCTGAAACAGCTCATGGTAAGGTTGCA
AACTATATTCCGATATTAGGAATTGTTGAT
CCGCAGCAACTGGGCATTGCCATTTATGAC
GTTGACGAGGATGAAATTGGGACCGCTGGA
ATGGCTGGAACGCGATTTGCAATTGAGAGT
ATTGCAAAGGTCGTTGCCTTAATATTAGCG
ATCAAGAGATTAGGACATGAGCGTGTCTTA
GCAGAACTGGAAAATGGTTCGGCAGATTAT
AGCCTAAGTTCGGTACTATTGGATGATGAG
TTGACCGAGCAAGCACACCGAGTAAATTAC
CTTAATAATTCCTCCGCCTTGTTAACGACA
GCCTTAATTGACCAGTTGATGGGTCAAAAT
AGTTTTAATGCTCTTCTCGGTTTCTGCCGG
GAGATCTGCAATGACCCATGTATCAGTTTG
AATGAGCGTTTGTTTCGATCGGCAATTATG
AATGATAAAGATATTCATGCACTGGCTTAC
TATATGAAAGATAAGGATATTTTAGAGACT
GTTGATCAAACATTGATAACTTACTTTATG
CAAAGCTCAATGATGGTGACATCACAGAGT
CTGGCTAACTTAGGAGCAGTCTTGGCAAAT
GATGGAATTAAACCTTGGAATAATGAGCGT
CTTATTAGCCATGAAGATAACGAGTTGGTA
AAGAAATTGCTAACAACAGTTGGTTCGTTT
GAAGAATCAAAAGAATACACAATTAAAATT
GAACTCCCTATTAAAAGTGGTACTGGCGGT
GGCTTATTGGCTTGTGCCCCGCAAAAATGT
GGTATTGGTATTTTTAGTCCAGCTCTTGAT
CAACATGGCAATAGTTTGGCAGGAATGAGT
TTATTACAAGATGTTGTTGATCAATTAGAA
TTAGTAGTTTAA

E. coli JM109 pJRS pKO-*gls*2-AB

Primers

gls2-KO1-BamHI

CGGGATCCTTGCCGATGCATTAAC

gls2-KO2-XbaI

GCTCTAGACtATTGCTCTAATTTTTGCATCGT

gls2-KO3-XbaI

GCTCTAGATTAGAATTAGTAGTTTAATAAAA
GCG

gls2-KO4-PstI

AACTGCAGGGAAACGCAGATGAGAG

Amino acid sequence Gls 2; 304 aa

MQKLEQLIDKNFAETAHGKVANYIPILGIVDPQQLGIAIYDVD
EDEIGTAGMAGTRFAIESIAKVVALILAIKRLGHERVLAELEN
GSADYSLSSVLLDDELTEQAHRVNYLNNSSALLTTALIDQLMG
QNSFNALLGFCREICNDPCISLNERLFRSAIMNDKDIHALAYY
MKDKDILETVDQTLITYFMQSSMMVTSQSLANLGAVLANDGIK
PWNNERLISHEDNELVKKLLTTVGSFEESKEYTIKIELPIKSG
TGGGLLACAPQKCGIGIFSPALDQHGNSLAGMSLLQDVVDQLE
LVV

gls3

> In Marcia and Sabine's lab books, it is 71323 or 323 referred to 2500071323 L-glutaminase;. For publications, it should be *gls3*

>921 bp

ATGAATCTTAATGACGCAATTCAAGAATGT
TGGAGCAAAATTGACGAGGGACAGGTAGCA
ACCTATATTCCTGCTTTAGCAAAAGTTGAC
CCTTATCAACTTGGCGTTTACCTTTTTGAC
GTCACAAATGATAAAAAAGTAGAAGCAGGC
GCATCGCAAGTTCGGTTTGCTATCGAAAGT
GTCTCGAAAGTAATCACCCTCCTCTACGCA
ATTGAACGTTTAGGCTTGTCAGCAGTTGAA
GAGCAAGTCGGAACACGCCAGACCGGCTTC
CCGTTCGATACAATCCTTAACATGGAAATC
ACTAAAGAGACCCACCCTCTCAATGCTTTT
ATTAATAGTGGTGCCATCCTCATCAGTTCA
TTAATCGAAGAACAAGATGGGCTTTCTCCC
TTTGACCAAATCCTTGAATTTAGTCGTAAA
ATTTGCAATGATCCTGATATCACCCTCAAT
GAAGAAATTTACCAATCGGAGTTGCGAACC
GGGGATATGAATCGGTCGTTAGCCTACTAT
CTCAAGGCCAAAGAAGTCCTCACTAATGAT
GTAACCCTTAGCCTTGATACCTATTTTAAG
CAGTGTTCAATGATGGTCACTTGCCAAAGT
CTTGCTAACCTCGGTGCTGTCCTTGCCAAT
GACGGGATCGCTCCTTGGAATAATGAACGA
ATCATTTCAAGCGAGGCTGCTACATATACA
AAATCGGTCATGATGACGACTGGCCTCTAC
AATGAATCAGGAACTTATTCCGTTCGAATC
GGTGTACCGACTAAGAGCGGTGTCGGTGGC
GGTTTAGTTTCTGCTGCCCCCAATCATTAC
GGGATCGGAATTTTCAGTCCGGCACTCGAC
CATGCTGGTAATAGTGTTGCCGGGCTAGCA
CTCCTTGAACTCATCAGTAAAAAATTAAAG
CTTGATATTTTTAGGTACTAG

E. coli JM109 pJRS pKO-*gls*3-AB

Primers

gls3-KO1-PstI

AACTGCAGAAAAGCTTGGACAACCC

gls3-KO2-EcoRI

GGAATTCTTATTTAAGATCCAAAGTAATCACCTC

gls3-KO3-EcoRI

GGAATTCTTTCAGTACTAATAATTAAGGTCCAA

gls3-KO4-BamHI

CGGGATCCGCATGTGCTGAAAATTG

Amino acid sequence Gls 3; 306 aa

MDLKQVVDNNLAKTNLGKVANYIPALGKVDPKQLGIAVYDLNK
DNITNAGNADVRFAIESISKVIVFLYAAKQLGLSNVTKHVGAR
QTGFPFNSILNMEIEKAHYPLNPFINAGAIEVTSLIQPYNGKS
PFDQIIQFAREICHDPQISLNSEIFESEDRTGDTDRSLAYYLK
ANKMIHADVTTSLTTYFKQCSMMVTAISLANLGAVLANNGIKP
WDNKRLVPTEVATYTKSLMTTTGLYNESGTYSSIIGVPTKSGV
GGGLMSAAPNRFGIGIFSPPLDNAGNSVAGLAALEEISNELKI
DIFQY

V. Sequencing results for *L. reuteri* 100-23 Δ*gls*2-3 colony #4 and #5 (Figure1)

L. reuteri 100-23 Δ*gls*2-3 colony #4 > Primer gls3-F 914bp

NNNNNNNNNNTNNNNNNNNNATTTCNNTCNCCNCNAGTCNNTNTCTACCA
TACAATTAATTTATTTATCAAATAAATAAGCTTGAATAAGCTTTTGTGTC
CAAAAATAGACTTATTGCTTGTCGTTTTATATTTATAACAGTATCCTAGC
ATTTAGGAGGTGATTACTTTGGATCTTAAATAAGAATTCTTTCAGTACTA
ATAATTAAGGTCCAACTCATACTAAATTCTTAACAAATGATTTTCTTACT
TAAGAATCAATTTTTATCATTCGAGATATCATGAATGAGTCATAATATAG
TTACAAATCCCCTTGATAATTGTAATGAATTATTGAGGGGATTTATGTTT
ATGAGATCAAATATAGAATTTTAATATGTATGATTAAAACAGAAACTAAA
AAATAATTAGCAATTAATATAATTTTGCAATTTTTACAGTAAAAAGTANN
NNNCCCGGCTANCGGGGGGCGGGTGGGTTTTTAAAAGGGGGGGGGGGGGG
GGAGAAAAGGGGGGGGGGGGGGGAAAAAAAAAGCCCGNNTAAATTTCAA
AAGGTGGACCCCNNAAAAAAAAAATCCCCTCAATAATTCATTACAATTATC
AAGGGGGTTTNNNACTNTATTATGACTNATTCNNGAAANCCCGANNGNAA
AAAATNGGTTCTAAGGAAAAAAACCTTTTTTAANGAATTTAGGNTGGGTN
GGCCTAAATTTTTGGTNCTAAAAAATTTTTTTTTTTAGAACCNAGNAACCC
CCCCCAAANCNGGGNNNNTTTNNAAANNANAANNCANGANAAAGNTTTTT
TTTGNNNNNAAANTTTTNNNNNTTTTTTTTTNNNAAAAAAANNNTTTNNG
GGGAAAANNNGGGGGGNGNNNAAAATTTTTTTNNNAAAAGCGNNNNNNGN
GNNNTTNNAAAAAN

L. reuteri 100-23 Δ*gls*2-3 colony #4 > Primer gls3-R 466bp

NNNNNNNNNNNNNNNANNTNNTGNNNNNANNNNNNNNGTATAGTTTCNNN
NTTTAATCATACATATTAAAATTCTATATTTGATCTCATAAACATAAATC
CCCTCAATAATTCATTACAATTATCAAGGGGATTTGTAACTATATTATGA
CTCATTCATGATATCTCGAATGATAAAAATTGATTCTTAAGTAAGAAAAT
CATTTGTTAAGAATTTAGTATGAGTTGGACCTTAATTATTAGTACTGAAA
GAATTCTTATTTAAGATCCAAAGTAATCACCTCCTAAATGCTAGGATACT
GTTATAAATATAAAACGACAAGCAATAAGTCTATTTTTGGACACAAAAGC
TTATTCAAGCTTATTTATTTGATAAATAAATTAATTGATGGTAGAAATAG
CTAGGGGAGAAGAAATGAATTTTTTATGAAAGGAAGCGCGNGAGNNNGNN
NTNNNNNNAAAGGANN

L. reuteri 100-23 Δ*gls*2-3 colony #5 > Primer gls3-F 459bp

GNNNNNNNNNNANNNNNNNTNNTNNTCTTCTCNNCNAGCTATTTCTACCA
TCAATTAATTTATTTATCAAATAAATAAGCTTGAATAAGCTTTTGTGTCC
AAAAATAGACTTATTGCTTGTCGTTTTATATTTATAACAGTATCCTAGCA
TTTAGGAGGNGATTACTTTGGATCTTAAATAAGAATTCTTTCAGTACTAA
TAATTAAGGTCCAACTCATACTAAATTCTTAACAAATGATTTTCTTACTT
AAGAATCAATTTTTATCATTCGAGATATCATGAATGAGTCATAATATAGT
TACAAATCCCCTTGATAATTGTAATGAATTATTGAGGGGATTTATGTTTA
TGAGATCAAATATAGAATTTTAATATGTATGATTAAAACAGAAACTAAAA
AATAATTAGCAATTAATATAATTTTGCAATTTTTACAGAAAAAAGTAGCA
CACCAGCTA

L. reuteri 100-23 Δ*gls*2-3 colony #5 > Primer gls3-R 458bp

NNNNNNNNNNNNNNNNNNNTTNNTGTNNNNANNNNATGNNTGTATATGTT
TGCATGTTTTAATCATACATATTAAAATTCTATATTTGATCTCATAAACA
TAAATCCCCTCAATAATTCATTACAATTATCAAGGGGATTTGTAACTATA
TTATGACTCATTCATGATATCTCGAATGATAAAAATTGATTCTTAAGTAA
GAAAATCATTTGTTAAGAATTTAGTATGAGTTGGACCTTAATTATTAGTA
CTGAAAGAATTCTTATTTAAGATCCAAAGTAATCACCTCCTAAATGCTAG
GATACTGTTATAAATATAAAACGACAAGCAATAAGTCTATTTTTGGACAC
AAAAGCTTATTCAAGCTTATTTATTTGATAAATAAATTAATTGATGGTAG
AAATAGCTAGGGGAGAAGAAATGAATTTTTTATGNAAGGAAGCGNGTGNNNNCATGTN

VI. Sequencing results for *L. reuteri* 100-23 Δ*gls*1-2-3 colony #7 and #8 (Figure1)

L. reuteri 100-23 Δ*gls*1-2-3 colony #7 >Primer gls1-F 540bp
NNNNNNNNNANTGCATCNNTNNNNNNNNNNNNNNNNTATAACCGGTAGCT
GGATCAATGATAAAGCAGTACCTTTTCAACGATTTGCGGTTAAAACCCAT
GCTTATGAAGGCGGCGTTTCTCGTCTAGAAGATGGCGAAGAAGTAGATTA
CGAGTTTATTGCTGATGCATATACTGGTAGTCTATTAGAATTAAAACGGA
TTGAGAATAACTAGAGCAAAGGCTGGGGACAAATTTCCCAGCGTTTTTAA
TTAAAGGAGGATATCATGAATCTTAATGACGCAATTCAAGAATGAAGATC
TAGGTACTAGTTGCAAATATTCGCCTCCTTATCGGGATCTACTTAATTAT
ATTCAACGTTCAATCCAGTTTCGTTAATTTACTATTCCAATTCAATATTC
AGGTACATACCAAGAACATTTTTAGTATAATTAAGGCAATTGATGCATAC
GCTTTCACCAATAAGGAGGTTTCTCATGATGTATCCGGAAACAATCGGTC
ATTCTTAAGNTACAGTAAAACNCCTTTGTNNAAANNNAAC

L. reuteri 100-23 Δ*gls*1-2-3 colony #7 >Primer gls1-R 553bp
NNNNNNNNNTNNNTCNGATCNGNNGANNNNTNNNNNANNATACATCATGA
GAAACCTCCTTATTGGTGAAAGCGTATCGCNTCAATTGCCTTAATTATAC
TAAAAATGTTCTTGGTATGTACCTGAATATTGAATTGGAATAGTAAATTA
ACGAAACTGGATTGAACGTTGAATATAATTAAGTAGATCCCGATAAGGAG
GCGAATATTTGCAACTAGTACCTAGATCTTCATTCTTGAATTGCGTCATT
AAGATTCATGATATCCTCCTTTAATTAAAAACGCTGGGAAATTTGTCCCC
AGCCTTTGCTCTAGTTATTCTCAATCCGTTTTAATTCTAATAGACTACCA
GTATATGCATCAGCAATAAACTCGTAATCTACTTCTTCGCCATCTTCTAG
ACGAGAAACGCCGCCTTCATAAGCATGGGTTTTAACCGCAAATCGTTGAA
AAGGTACTGCTTTATCATTGATCCAGCTACCGGTTAAAGAACCTTCACTT
GCAAAATCGTTTTTGACTAATNTTAAGATCNNANANNNNGNNNTTTTGGG
NAA

L. reuteri 100-23 Δ*gls*1-2-3 colony #8 >Primer gls1-F 545bp
NNNNNNNNNNNNNANNNNTTTGCAGTGAGNTTCTTTAACCGGTAGCTGGA
TCAATGATAAAGCAGTACCTTTTCAACGATTTGCGGTTAAAACCCATGCT
TATGAAGGCGGCGTTTCTCGTCTAGAAGATGGCGAAGAAGTAGATTACGA
GTTTATTGCTGATGCATATACTGGTAGTCTATTAGAATTAAAACGGATTG
AGAATAACTAGAGCAAAGGCTGGGGACAAATTTCCCAGCGTTTTTAATTA
AAGGAGGATATCATGAATCTTAATGACGCAATTCAAGAATGAAGATCTAG
GTACTAGTTGCAAATATTCGCCTCCTTATCGGGATCTACTTAATTATATT
CAACGTTCAATCCAGTTTCGTTAATTTACTATTCCAATTCAATATTCAGG
TACATACCAAGAACATTTTTAGTATAATTAAGGCAATTGATGCATACGCT
TTCACCAATAAGGAGGTTTCTCATGATGTATCCGGTAACAATCGGTCATT
CAGAAGTTAAAGNNAATCCCCTCAANNNNGGANANNNNCTGTTAA

L. reuteri 100-23 Δ*gls*1-2-3 colony #8 >Primer gls1-R 550bp
NNNNNNNNNNNNNNNNNTCTGATGACCGATTGTTACCGGATACATCATGA
GAAACCTCCTTATTGGTGAAAGCGTATGCNTCAATTGCCTTAATTATACT
AAAAATGTTCTTGGTATGTACCTGAATATTGAATTGGAATAGTAAATTAA
CGAAACTGGATTGAACGTTGAATATAATTAAGTAGATCCCGATAAGGAGG
CGAATATTTGCAACTAGTACCTAGATCTTCATTCTTGAATTGCGTCATTA
AGATTCATGATATCCTCCTTTAATTAAAAACGCTGGGAAATTTGTCCCCA
GCCTTTGCTCTAGTTATTCTCAATCCGTTTTAATTCTAATAGACTACCAG
TATATGCATCAGCAATAAACTCGTAATCTACTTCTTCGCCATCTTCTAGA
CGAGAAACGCCGCCTTCATAAGCATGGGTTTTAACCGCAAATCGTTGAAA
AGGTACTGCTTTATCATTGATCCAGCTACCGGTTAAAGAACCTTCACTTG
CAAAATCGTTTTTGACTAATTTTAAGATCCGATCANANNNNTTTGGGAAN