University of Alberta

Role of metabolic pathways in *Lactobacillus* ecology and food quality

by

Januana Souza Teixeira

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

©Januana Souza Teixeira

Fall 2013

Edmonton, Alberta

Permission is hereby granted to the University of Alberta Libraries to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only. Where the thesis is converted to, or otherwise made available in digital form, the University of Alberta will advise potential users of the thesis of these terms.

The author reserves all other publication and other rights in association with the copyright in the thesis and, except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatsoever without the author's prior written permission.

ABSTRACT

Lactobacilli constitute the natural microbiota of cereal fermentations, and their competitiveness has been attributed to the formation of organic acids and various antagonistic compounds. However, these traits alone do not fully explain the prevalence of specific *Lactobacillus* strains in cereal fermentations. This research demonstrated that the regulation of carbohydrate metabolism and amino acid-based acid resistance contribute to the competitiveness of the obligate heterofermentative *Lactobacillus reuteri* in cereal substrates.

The role of α-galactosidase, sucrose phosphorylase, and levansucrase for the conversion of raffinose family oligosaccharides was elucidated. It was shown that levansucrase contributes to the metabolism of raffinose family oligosaccharides, and allows the intermediate accumulation of α-galactooligosaccharides as prebiotic compounds. Further studies on the regulation of levansucrase and sucrose phosphorylase demonstrated that these enzymes are induced by sucrose or raffinose, but not repressed by glucose. Regulation is mediated by ScrR; deletion of *scrR* in *L. reuteri* resulted in constitutive expression of sucrose phorphorylase and levansucrase. The lack of carbon catabolite repression of sucrose metabolic enzymes in *L. reuteri* differentiates this organism from other lactobacilli and likely reflects adaptation to plant substrates.

Analysis of the glutamine / glutamate dependent acid resistance demonstrated that glutamine deamidation increased acid resistance independent of glutamate decarboxylation. Remarkably, glutamate decarboxylation not only

increased the intracellular pH. Electrogenic substrate / product antiport also polarized the membrane. Glutamate decarboxylation thus contributed to both components of the transmembrane proton motive force.

Improved knowledge of the acid resistance of *L. reuteri* allows a better understanding of *L. reuteri* to cereal and intestinal ecosystems, and facilitates the selection of strains that can be used as both starter and probiotic cultures.

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. M.G. Gänzle for his continuing support and guidance throughout my graduate study and research. His knowledge, supervision and encouragement made this thesis possible. It has absolutely been a privilege to have you as supervisor and I am deeply grateful for your support in all aspects of the PhD program. I am also thankful to my supervisory committee members Dr. L.M. McMullen and Dr. D.C. Bressler for their valuable input throughout these years, as well as members of my examining committee Dr. Lisabeth Truelstrup Hansen and Dr. Dominic Sauvageau for all of your time and support. Thank you also to my mentors, Dr. C. Schwab and Dr. Shu-Wei, for providing me with suggestions on where to take my research further and for their great contribution to this work. Thank you for the example of dedication and integrity that you have set.

Science has to be exciting and fun to be worthwhile. It is easier to achieve this when one is surrounding by enthusiastic colleagues who willingly dedicate their free time to tackling some of the most intriguing questions. I thank the present and former members of AgFor 2-50 for their enthusiasm and readiness to share their knowledge. While we were all doing our own things, having your support and friendship has meant a great deal during both the good times and those when things haven't gone so well. I am also thankful to the ones who came into the lab to work under my supervision and with whom I learned so much, namely Xiaoxi Lin, Victoria McNeill, Lucilla Dei Piu, Arisha Seeras and Tiange Shi.

Lastly, a warm thanks to my family and friends spread all over the globe, including the ones who are no longer present, for somehow always being there when I needed them the most. My gratitude to my dearest mother, my beloved father who unforutantely die much too yunger for both of us and whom I miss terribly, and my siblings for their unconditional love and support throughout this long and continuing journey in reaching my professional aspirations. A very special word of gratitude goes out to my fiancé, Tucaaue Alvares, who brings a great joy of life and who makes me want to be a better person. I am very thankful for his love, help and strong support in many difficult times.

TABLE OF CONTENTS

LIST OF TABLES

II. LIST OF FIGURES

III. LIST OF SYMBOLS AND ABBREVIATIONS

α-Gal	Alpha-galactosidase
α-GOS	Alpha-galactooligosaccharides
ADI	Arginine deiminase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
cDNA	Complementary DNA
cFDASE	Carboxyfluorescein diacetate succinimidyl ester
cFSE	Carboxyfluorescein succinimidyl ester
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EPS	Exopolysaccharides
FOS	Fructo-oligosaccharides
FtfA	Fructansucrase A
FUA	Food Microbiology culture collection of University of Alberta
GABA	γ-aminobutyric acid
GAD	Glutamate decarboxylase
GOS	Galacto-oligosaccharides
HPAEC-IPAD	High performance anion-exchange chromatography and integrated pulsed amperometric detection
HPLC	High performance liquid chromatography
KO	Knockout
LAB	Lactic acid bacteria
LB	Luria-Bertani broth
LevS	Levansucrase
LTH	Institut für Lebensmitteltechnologie, Universität Hohenheim
mMRS	Modified deMan-Rogosa-Sharpe medium
MRS	deMan-Rogosa-Sharpe medium
NAD	Nicotinamide adenine dinucleotide
PCR	Polymerase chain reaction
P_i	Inorganic phosphate
PTS	Phosphotransferase system
qPCR	Quantitative polymerase chain reaction

RFOs	Raffinose family oligosaccharides
RNA	Ribonucleic acid
ScrP	Sucrose phosphorylase
ScrR	Sucrose regulator
TMW	Technische Mikrobiologie Weihenstephan, Technische Universität München

CHAPTER ONE: **INTRODUCTION**

CHAPTER 1

1.1. Role of lactobacilli in food fermentation

Lactic acid bacteria (LAB) play an essential role in fermentation processes involving diverse food types such as milk, meat, vegetables, and fruits (Bourdichon *et al.*, 2012). The use of LAB in food fermentations can be traced back to ancient times as a method of preservation (Hammes and Hertel, 2006). These bacteria constitute a heterogeneous group of microorganisms with the formation of lactic acid as the sole or main metabolite of carbohydrate fermentation as a common metabolic property (Stiles and Holzapfel, 1997). The order *Lactobacillales* contains the majority of bacteria classified as LAB (Makarova *et al.*, 2006), including the genera *Weissella, Tetragenococcus, Streptococcus, Oenococcus, Pediococcus, Leuconostoc,* and *Lactococcus* which are relevant in food fermentations. However, some authors also consider the genus *Bifidobacterium* as part of LAB, despite the lack of physiological and phylogenetic relation with *Lactobacillales*. Metabolites formed during fermentation by LAB generally determine the flavor, texture and nutritional value of fermented food products. They also prolong the shelf-life of fermented foods, by preventing the growth of pathogenic and spoilage microflora. The genus *Lactobacillus* is frequently used for the production of such foods (Leroy and De Vuyst, 2004).

Lactobacilli can be found in a wide diversity of fermented foods, particularly in dairy products such as yogurt, cheese, and fermented probiotic milk. Additionally, fermentation of vegetable products such as sauerkraut, pickled vegetables, the Korean kimchi and the Caucasian kefir, also use *Lactobacillus*

spp. (Leroy and De Vuyst, 2004; Hammes and Hertel, 2006). These bacteria are also used in the production of fermented dry sausages and other meat-derived commodities. In alcoholic drinks such as beer and wine, lactobacilli may contribute to the flavor, but may also act as contaminants (Shalaby, 1996; Leroy and De Vuyst, 2004).

It is noteworthy that lactobacilli also occur in sourdough and related cereal fermentations (Hammes *et al.*, 2005; Hammes and Hertel, 2006). Sourdough is an intermediate product in bread production, in which the microflora is composed mainly of lactic acid bacteria and yeast. The nutrient availability ultimately modulates the microbial ecology of sourdough, and *Lactobacillus* species are the most frequently observed genus (De Vuyst *et al.*, 2013). In addition to the positive effects on sourdough fermentation by *Lactobacillus* species on bread characteristics such as flavor, texture, and shelf life, lactobacilli sourdough strains also contribute to its functional value, by producing compounds with low glycemic index, dietary non-digestible oligosaccharides and fiber. Moreover, they also promote vitamin enhancement and increase the bioavailability of minerals, as recently reviewed by Katina and Poutanen (2013).

In addition to their use in food fermentations, lactobacilli have a role as probiotics – live microorganisms which, when present in adequate amounts, confer a health benefit to the host (Sanders, 2008). In both humans and animals, *Lactobacillus* species are commonly found among the resident microbiota of the gastrointestinal and genitourinary tracts; their concentrations will vary with host age and dietary habits (Hammes and Hertel, 2006). In these environments,

lactobacilli establish several interactions with the host and have been linked with numerous health benefits, such as immunomodulation, intestinal integrity, and pathogen resistance (Schiffrin and Blum, 2001). Interestingly, lactobacilli found in sourdough also inhabit human and animal intestines, namely *Lactobacillus reuteri* and *Lactobacillus acidophilus* (Vogel *et al.*, 1999). Furthermore, some sourdough lactobacilli, particularly *L. reuteri*, originate from intestines of vertebrates (Su *et al.*, 2012).

1.2. Keys to lactobacilli success in food fermentation

1.2.1. Metabolic adaptability

Members of the genus *Lactobacillus* occupy numerous different ecological niches in nature. Classified as nutritionally fastidious organisms, they are widespread in nutrient-rich environments. Lactobacilli are often found in very diverse habitats ranging from plants and fermented products to the mucosal surface of humans and animals, where carbohydrates and proteins are usually abundant (Hammes and Hertel, 2006).

Lactobacilli rely on the availability of easily fermentable sugars, amino acids, vitamins and nucleotides to grow very fast, with duplication times of less than an hour. Regarding their mineral requirements for growth, *Lactobacillus* species are generally considered to be iron abstainers. In many bacterial species, iron acquisition is associated with respiration, and it is an indispensable co-factor for many cellular processes. In pathogenic enterobacteria, this physiological characteristic plays an essential role in their virulence and colonization (Litwin and Calderwood, 1993). Lactobacilli are considered to be an exception among

living organisms because they do not require iron for growth (Weinberg, 1997). Subsequently, this might represent an ecological advantage in a low-iron concentration environment, where they compete with pathogenic bacteria (e.g. intestinal tract) (Chung *et al.*, 1998; Engels *et al.*, 2011).

The efficient use of nutrients and the concomitant production of lactic acid during their growth provide these organisms with remarkable selective advantages in the diverse ecological niches they inhabit. Monosaccharides or oligosaccharides are the primary source of metabolic energy for lactobacilli (Hammes and Hertel, 2006). The type of carbohydrates and microorganisms present in fermented foods result in different end-products, which contribute not only to the development of the overall sensory properties (helping to determine unique product characteristics) but also to the preservation of fermented foods.

Because of their close association with humans, food animals, and food, and their consequent economic importance, *Lactobacillus* species have been studied for more than a century and as a result, a substantial amount of information on their genetic, physiology and metabolism is available (Kandler, 1983; Axelsson, 2004; Makarova *et al.*, 2006; Gänzle *et al.*, 2007; Gänzle and Follador, 2012).

Based on their main carbohydrate fermentation pathways – glycolysis and pentose phosphate pathways – lactobacilli can be grouped into homofermentative, facultative heterofermentative, and obligate heterofermentative (Axelsson, 2004). Homofermentative lactobacilli metabolize hexoses via basic glycolysis to yield lactic acid as the major metabolic end product. Species that belong to this group

can be shifted to produce alternative metabolites from glycolysis, including compounds such as formate, acetate, ethanol and CO_2 in addition to lactate. The facultative heterofermentative lactobacilli (e.g., *Lactobacillus plantarum*) degrade hexoses via glycolysis, yielding lactate as major end product, and degrade pentoses via pentose phosphate pathway, yielding lactic acid and acetic acid. In these organisms, carbon sources are used in a hierarchical manner and genes necessary for catabolizing the less preferred substrate are repressed, in a mechanism known as carbon catabolite repression (CCR) (Titgemeyer and Hillen, 2002). Species grouped as obligate heterofermentative degrade hexoses as well as pentoses via the pentose phosphate pathway in a strain-dependent ratio of lactic acid, acetic acid, carbon dioxide and ethanol (Hammes and Vogel, 1995).

Heterofermentative metabolism of hexoses has a poor energy yield when compared to homofermentative metabolism. This disadvantage may be compensated by the presence of external electron acceptors (e.g., oxygen, fructose, glycerol), which create alternative pathways for NADH reoxidation, that were generated in the upper branch of the pentose phosphate pathway, resulting in gain of additional energy and increased growth rates (Stolz *et al.*, 1995a; Stolz *et al.*, 1995b). Adaptation to environments where external electron acceptors are present may lead to an efficient ATP/glucose ratio making the pentose phosphate pathway the dominant pathway.

1.2.2. Acid resistance

The conversion of fermentable carbohydrates to lactic acid and other organic acids by lactobacilli imparts a unique flavor to fermented products but

also causes acidification of the environment, and therefore challenges the microflora, inducing an increase in its acid resistance (Leroy and De Vuyst, 2004). Lactobacilli thus need to respond to acidification in order to ensure physical and genetic integrity by relying on the involvement of multiple acid resistance mechanisms for survival. Understanding the mechanisms involved in such acid adaptation leads to selection of strains to improve flavor, texture and also the nutritional value of specific foods.

Acid stress has been extensively studied in the facultative heterofermentative *Lactococcus lactis* and *L. plantarum*, species which prevail in dairy products and in a wide variety of plant substrates (De Angelis and Gobbetti, 2004). *L. plantarum* is the leading species in the stress physiology research of the genus. Hitherto, little is known about obligate heterofermentative species and their response to acid stress.

Among the obligate heterofermentative lactobacilli, *L. reuteri* is of particular relevance because strains of this species inhabit the upper intestine of rodents, pigs, and poultry (Reuter, 2001; Walter, 2008) and also prevail in sourdough fermentations that are carried out at elevated fermentation temperatures with long incubation times, thus selecting for increased acid tolerance of the bacterial cultures (Vogel *et al.*, 1999; Meroth *et al.*, 2003). Furthermore, this species also has been used in probiotic foods (Smits *et al.*, 2005). Growth and survival in these diverse environmental niches relies on the ability of the organism to sense and respond to varying conditions such as nutrient availability and pH. Due to these reasons, *L. reuteri* has attracted interest in

studies aiming to understand its enhanced physiological flexibility, and can be used as a suitable model organism for the evaluation of genetic and metabolic traits important for competitiveness in the gut and also in different food ecosystems.

1.3. Carbohydrate metabolism

The variable sources of fermentable carbohydrates in food ecosystems will determine different physiological behaviors by lactobacilli, including the preferential or simultaneous use of energy sources, regeneration of reduced cofactors, and the interaction with exogenous or endogenous enzymes from the ecosystem (Vogel *et al.*, 1999; Gänzle *et al.*, 2007). The high prevalence of obligate heterofermentative *Lactobacillus* species in cereal fermentations is based in part on the simultaneous consumption of several sugars combined with the regeneration of reduced cofactors, which helps to compensate for the poor energetic yield of their hexose metabolism (Gänzle *et al.*, 2007).

Sucrose is widespread in plants and is the most abundant sugar in many fruits, grain legumes, and ungerminated cereal grains, which makes it a major energy source for heterofermentative *Lactobacillus* strains often found in cereal fermentation. The ability to metabolize sucrose contributes to the ecological fitness of lactobacilli in sourdough fermentations (Vogel *et al.*, 1999). Lactobacilli express several alternative enzymes and transport systems involved in sucrose metabolism (for review, see Gänzle and Follador, 2012). Sucrose metabolism is mediated by (phospho-)fructo-furanosidase catalyzing sucrose hydrolysis; which

is frequently associated with sucrose phosphotransferase systems (PTS) and likely also recognizes sucrose phosphate as substrate. Additionally, an intracellular sucrose phosphorylase (ScrP) or extracellular glycansucrases (glucan- and fructansucrases) complement or substitute the (phospho-)fructo-furanosidase enzyme in a few *Lactobacillus* spp. Recent analysis of 38 lactobacilli genomes, with the exception of *Lactobacillus brevis*, demonstrated that genomes harbor at least one functional sucrose metabolic pathway, indicating that sucrose is a highly preferred substrate (Gänzle and Follador, 2012). Metabolic enzymes for sucrose conversion partially overlap with the metabolism of fructo-oligosaccharides and raffinose-family oligosaccharides. Sucrose is the only substrate for glucansucrases, while fructansucrases also can use raffinose (Van Geel-Schutten *et al.*, 1999). According to Trindade *et al.* (2003) and Kullin *et al.* (2006), expression of ScrP is induced by sucrose as well as by raffinose.

L. reuteri generally expresses several glycansucrases, which use sucrose as glycosyl donor for the production of exopolysaccharides (EPS) as alternative to sucrose hydrolysis (Van Geel-Schutten *et al.*, 1999; Schwab *et al.*, 2007; Kaditzky *et al.*, 2008). These long-chain EPS contribute to the resistance of lactobacilli to chemical and physical stressors and also have technological relevance in food products, i.e. can act as hydrocolloids (Gänzle and Schwab, 2009; Galle and Arendt, 2013). *L. reuteri* also harbors the intracellular sucrose phosphorylase enzyme (ScrP), which breaks down sucrose to glucose-1-phosphate and fructose as an energetically favorable alternative to sucrose turnover by glycansucrases (Schwab *et al.*, 2007). Fructose released from sucrose metabolic enzymes can also

be used as an electron acceptor and reduced to mannitol, through mannitol dehydrogenase enzyme, to achieve co-factor regeneration allowing to the gain of additional ATP (Stolz *et al.*, 1995b). The presence of alternative pathways for sucrose metabolism may reflect importance of sucrose metabolic enzymes, and further understanding on their regulation may enhance our ability to control and use these bacteria in the food industry.

1.4. Mechanisms of acid resistance in lactobacilli

Lactobacilli are confronted with acidic conditions, generated during fermentation, or inhabiting acidic environments such as animal and human intestines. Both the physiological status of the cells and environmental factors influence the acid stress response (for reviews, see Konings, 2002; Cotter and Hill, 2003; De Angelis and Gobbetti, 2004). The mechanisms involved in acid resistance in lactobacilli share significant similarities with the acid resistance in *Escherichia coli*. *E. coli* has evolved different strategies to overcome acidic stress. Individual organisms exhibit a different degree of resistance to acid stress, involving acid resistance (AR) systems, which typically protect stationary-phase cells from an extreme acid stress ($pH \leq 2.5$) without pre-adaptation (Foster, 2004).

In lactobacilli, responses to acid stress are mediated by differential expression of stress proteins and by up-regulation of metabolic pathways that contribute to pH homeostasis (De Angelis and Gobbetti, 2004). Mechanisms include: F_0F_1-ATPase proton pumps, amino acid decarboxylation/deimination, synthesis of alkaline compounds, and modification of the cell membrane

composition (Cotter and Hill, 2003). Glutamate decarboxylation and arginine deiminase are non-specific acid resistance mechanisms that are employed almost universally by bacteria and Gram-positive bacteria, respectively (Cotter and Hill, 2003; Foster, 2004). Synthesis of alkaline compounds through urease activity and synthesis of EPS are examples of acid resistance mechanisms which are more specific to certain ecosystems (Dols-Lafargue *et al.*, 2008; Walter *et al.*, 2011).

1.4.1. F_1F_0-ATPase

The proton-translocating ATPase (F_0F_1-ATPase) is a membrane-bound enzyme that catalyzes the translocation of H^+ concomitant with either the hydrolysis or synthesis of ATP. Considered an important homeostatic system for fermentative bacteria, this multimeric enzyme has a higher hydrolysis activity at low pH and is therefore crucial for the maintenance of ΔpH in these bacteria (Hutkins and Nannen, 1993; Konings, 2002).

1.4.2. Urease-based acid resistance

Urease catalyzes the conversion of urea into CO_2 and ammonia, which can alkalinize the pH and facilitate survival (Mobley *et al.*, 1995). The urease gene cluster is highly conserved among rodent *L. reuteri* isolates, while rare in porcine isolates and absent in human and poultry isolates (Walter *et al.*, 2011). A high proportion of urease-positive strains in isolates from a given ecosystem likely reflect a competitive advantage of these strains over urease-negative strains. In fact, in some rodent isolates of *L. reuteri* urease activity was found to contribute to acid resistance (Walter *et al.*, 2011; Wilson *et al.*, 2011).

1.4.3. Exopolysaccharides

In addition to the improvement of organoleptic and technological properties of several fermented foods, the synthesis of EPS by glycansucrases plays an important role in acid stress tolerance of EPS-forming strains (Gänzle and Schwab, 2009). *L. reuteri* strains generally use sucrose as a substrate for the production of EPS (e.g., glucan and fructan) to counteract the acid stress experienced during stationary phase (Kim *et al.*, 2000; Kaditzky *et al.*, 2008). Likewise, bacterial isolates producing β-glucan, which are often associated with wine or cider spoilage, survive better in an acidic environment compared to β-glucan negative strains (Dols-Lafargue *et al.*, 2008).

1.4.4. Amino acid-based acid resistance

Proteolysis carried out by enzymes from lactobacilli and the food matrix generate free amino acids, thereby enhancing the nutritional value and digestibility of the fermented food, as well as improving the acid resistance of the starter cultures (Gänzle *et al.*, 2008; Su *et al.*, 2011).

A number of systems involving amino acid decarboxylation or deamination coupled with precursor/product exchange form one of the most important bacterial strategies for surviving acid stress conditions. In particular, a precursor amino acid (e.g., histidine, arginine, and glutamate) is taken up from the medium, metabolized intracellularly, and excreted via a precursor/product exchange system into the medium as its amine derivative (for review, see Konings, 2002; Cotter and Hill, 2003).

The combination of amino acid conversion – antiport reactions can be seen as a general system for pH homeostasis, where the net effect is an increase in the alkalinity of the cytoplasm compartment. In addition to the increase of cytoplasmic pH, this precursor/product exchange can generate a translocation of charge across the cytoplasmic membrane, influencing the membrane potential ($\Delta\Psi$). This second component of the proton motive force (pmf) also provides the cell with metabolic energy, and contributes to microbial resistance under energy-limited environments (Konings, 2002).

1.4.5. Arginine deiminase

Another mechanism for the generation of alkali is the arginine-deiminase pathway (ADI). Arginine is decarboxylated to agmatine by *E. coli* and related organisms (Foster, 2004; Richard and Foster, 2004); whereas *Listeria monocytogenes* and lactobacilli, including *L. reuteri*, convert arginine to ornithine (Konings, 2002; Rollan *et al.*, 2003; De Angelis and Gobbetti, 2004).

In lactobacilli the degradation of arginine leads to the formation of ornithine, ammonia and CO_2, coupled to the production of ATP. The resulting NH_3 combines with H^+ in the cytoplasm to produce NH_4^+, helping to alkalize the environment. Furthermore, the extra energy (ATP) generated enables the extrusion of cytoplasmic protons by the F_0F_1-ATPase, and may help the cell to survive longer after depletion of the primary energy source. An arginine/ornithine antiporter completes the system, and catalyzes an electroneutral exchange of these two molecules at no energy cost, and contributes to the generation of proton

motive force (Konings, 2002; Cotter and Hill, 2003; De Angelis and Gobbetti, 2004).

This mechanism is widely conserved in lactobacilli, and available data strongly support their role in enhancing the acid resistance of these bacteria (De Angelis *et al.*, 2002; Rollan *et al.*, 2003; Dal Bello *et al.*, 2005). Additionally, the expression of the ADI pathway favors an increase synthesis of ornithine – a precursor compound of 2-acetyl-1-pyroline, a key flavor compound of the wheat bread crust formed during baking – and has been associated with improved bread flavor (Schieberle and Grosch, 1985; Thiele *et al.*, 2002).

1.4.6. Biogenic amines

A considerable amount of the research done on amino acid decarboxylation pathways is focused on the undesirable formation of biogenic amines (BA) in fermented foods matrices (Silla Santos, 1996; Fernandez and Zuniga, 2006; Spano *et al.*, 2010). The accumulation of biogenic amines as result of amino acid decarboxylase activity (e.g., histamine and tyramine resulted from decarboxylation of histidine and tyrosine, respectively) has been reported as a defense mechanism used by bacteria to counteract acidic environments (for review, see Konings, 2002; De Angelis and Gobbetti, 2004; Spano *et al.*, 2010). In the decarboxylation reaction, a proton is consumed, and CO_2 is released. The precursor uptake and excretion of the product across the membrane is mediated via an exchange mode of transport that is electrogenic (Konings, 2002). The excreted product carries one more positive charge than the precursor; thus the coupled reaction of decarboxylation and electrogenic exchange in the pathways

generate a proton motive force and provide the cell with secondary metabolic energy (Molenaar *et al.*, 1993; Wolken *et al.*, 2006). Synthesis of biogenic amines was shown to confer acid stress resistance through the histidine decarboxylation pathway of *Lactobacillus buchneri* (Molenaar *et al.*, 1993) and the tyrosine decarboxylation pathway of *Enterococcus faecium* (Pereira *et al.*, 2009) and *Lactobacillus brevis* (Wolken *et al.*, 2006).

1.4.7. Glutamate decarboxylase

One of the most effective amino acid-dependent acid resistance systems is the glutamate decarboxylase pathway (GAD). It has been proposed that its working model controls the cytosolic environment by decarboxylating an acidic substrate molecule (glutamate) into a neutral compound (γ-amino butyric acid, GABA) by consuming an intracellular proton. GABA would then be exported into the extracellular environment via a dedicated antiporter, GadC, while bringing in more glutamate, thereby contributing to alkalization (for a review, see Cotter and Hill, 2003).

Sanders *et al.* (1998) and Higuchi *et al.* (1997) proposed an intriguing alternative hypothesis for the function of GAD system, where by coupling intracellular glutamate decarboxylase-mediated proton consumption (GadB) with the electrogenic antiport carried out by GadC generates a proton motive force, which provides metabolic energy.

The GAD system in the enteric bacteria *E. coli* is one of the most intensively studied amino acid-dependent acid resistance system. Acid resistance has been reported in *E. coli* through the use of glutamate / GABA antiporters in

glutamate-supplemented media (Hersh *et al.*, 1996; Waterman and Small, 1996). This mechanism has been found in lactobacilli, where the ability to produce and release GABA has been linked to additional physiological functions (Coda *et al.*, 2010; Stromeck *et al.*, 2011). Siragusa *et al.* (2007) demonstrated the survival rate of GABA-producing strains of *L. plantarum* under simulated gastrointestinal conditions, as well the increase of functional value of fermented foods. Likewise, glutamate decarboxylase-mediated acid resistance was shown to contribute to the persistence of GABA-producing *L. reuteri* strains in sourdough fermentations (Su *et al.*, 2011).

1.4.8. Glutamine deamidase

Another mechanism that has received attention recently involves the deamidation of amino acid glutamine which is one of the most abundant amino acid in wheat proteins (Wieser, 2007). Glutamine uptake – likely to be mediated by GadC antiporter, as described by Ma *et al.* (2013) – is followed by its deamidation to glutamate through hydrolytic activity of glutaminases. This conversion also improves the adaptation of cereal-associated lactobacilli to fermentation acidity, which is attributed to the consumption of protons with concomitant release of ammonia, increasing the extracellular pH (Vermeulen *et al.*, 2007). Genomic characterization of glutaminase has been conducted for *E. coli* (Brown *et al.*, 2008). However, it has been only recently demonstrated that glutamine provides acid resistance for *E. coli* under acidic environment through enzymatic release of ammonia (Lu *et al.*, 2013). Identification of glutaminase has also been reported in cereal-associated lactobacilli *Lactobacillus sanfranciscensis*

and *L. reuteri* (Vermeulen *et al.*, 2007); however, their glutamine deamination pathway remains uncharacterized.

1.5. Research hypothesis and objectives

Lactobacilli constitute the natural microflora of cereal fermentations, and their competitiveness has been attributed to the formation of organic acids and various structurally different antagonistic substances; however, these traits alone do not fully explain the prevalence of specific *Lactobacillus* strains in cereal fermentation.

The experiments in this thesis were designed to test the hypothesis that regulation of carbohydrate metabolism and amino acid-based acid resistance contribute to the success of obligatory heterofermentative *L. reuteri* strains in cereal substrates. In doing so, this research aimed to contribute to a better understanding of the role of metabolic pathways in *Lactobacillus* ecology and food quality. Specific aims included:

I. Evaluation of the contribution of levansucrase and sucrose phosphorylase to raffinose, stachyose, and verbascose metabolism by lactobacilli;

II. Functional characterization of sucrose phosphorylase and *scrR*, a regulator of sucrose metabolism in *Lactobacillus reuteri*;

III. Analysis of mechanisms of acid resistance based on glutamine, glutamate, and arginine in *Lactobacillus reuteri*: are those redundant or complementary mechanisms?

1.6. References

Axelsson, L. (2004). "Lactic acid bacteria: classification and physiology." Lactic Acid Bacteria, Microbiology and Functional Aspects. S. Salminen, A. von Wright and O. A. New York, Marcel Dekker, Inc.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I. B., Prajapati, J. B., Seto, Y., Ter Schure, E., Van Boven, A., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S. and Hansen, E. B. (2012). "Food fermentations: microorganisms with technological beneficial use." Int J Food Microbiol 154(3): 87-97.

Brown, G., Singer, A., Proudfoot, M., Skarina, T., Kim, Y., Chang, C., Dementieva, I., Kuznetsova, E., Gonzalez, C. F., Joachimiak, A., Savchenko, A. and Yakunin, A. F. (2008). "Functional and structural characterization of four glutaminases from *Escherichia coli* and *Bacillus subtilis*." Biochemistry 47(21): 5724-35.

Chung, K. T., Lu, Z. and Chou, M. W. (1998). "Mechanism of inhibition of tannic acid and related compounds on the growth of intestinal bacteria." Food Chem Toxicol 36(12): 1053-60.

Coda, R., Rizzello, C. G. and Gobbetti, M. (2010). "Use of sourdough fermentation and pseudo-cereals and leguminous flours for the making of a functional bread enriched of gamma-aminobutyric acid (GABA)." Int J Food Microbiol 137(2-3): 236-45.

Cotter, P. D. and Hill, C. (2003). "Surviving the acid test: responses of gram-positive bacteria to low pH." Microbiol Mol Biol Rev 67(3): 429-53

Dal Bello, F., Walter, J., Roos, S., Jonsson, H. and Hertel, C. (2005). "Inducible gene expression in *Lactobacillus reuteri* LTH5531 during type II sourdough fermentation." Appl Environ Microbiol 71(10): 5873-8.

De Angelis, M. and Gobbetti, M. (2004). "Environmental stress responses in *Lactobacillus*: a review." Proteomics 4(1): 106-22.

De Angelis, M., Mariotti, L., Rossi, J., Servili, M., Fox, P. F., Rollan, G. and Gobbetti, M. (2002). "Arginine catabolism by sourdough lactic acid bacteria: purification and characterization of the arginine deiminase pathway enzymes from *Lactobacillus sanfranciscensis* CB1." Appl Environ Microbiol 68(12): 6193-201.

De Vuyst, L., Van Kerrebroeck, S., Harth, H., Huys, G., Daniel, H. M. and Weckx, S. (2013). "Microbial ecology of sourdough fermentations: Diverse or uniform?" Food Microbiol (in press).

Dols-Lafargue, M., Lee, H. Y., Le Marrec, C., Heyraud, A., Chambat, G. and Lonvaud-Funel, A. (2008). "Characterization of *gtf*, a glucosyltransferase gene in the genomes of *Pediococcus parvulus* and *Oenococcus oeni*, two bacterial species commonly found in wine." Appl Environ Microbiol 74(13): 4079-90.

Engels, C., Schieber, A. and Gänzle, M. G. (2011). "Inhibitory spectra and modes of antimicrobial action of gallotannins from mango kernels (Mangifera indica L.)." Appl Environ Microbiol 77(7): 2215-23.

Fernandez, M. and Zuniga, M. (2006). "Amino acid catabolic pathways of lactic acid bacteria." Crit Rev Microbiol 32(3): 155-83.

Foster, J. W. (2004). "*Escherichia coli* acid resistance: tales of an amateur acidophile." Nat Rev Microbiol 2(11): 898-907.

Galle, S. and Arendt, E. K. (2013). "Exopolysaccharides from sourdough lactic acid bacteria." Crit Rev Food Sci Nutr (just-accepted).

Gänzle, M. G. and Follador, R. (2012). "Metabolism of oligosaccharides and starch in lactobacilli: a review." Front Microbiol 3: 340.

Gänzle, M. G., Loponen, J. and Gobbetti, M. (2008). "Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality." Trends Food Sci Tech 19(10): 513-521.

Gänzle, M. G. and Schwab, C. (2009). "Ecology of exopolysaccharide formation by lactic acid bacteria: sucrose utilization, stress tolerance, and biofilm formation." Bacterial polysaccharides. M. Ullrich. Norfolk, Caister Academic Press: 263-278.

Gänzle, M. G., Vermeulen, N. and Vogel, R. F. (2007). "Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough." Food Microbiol 24(2): 128-38.

Hammes, W. and Hertel, C. (2006). "The genera *Lactobacillus* and *Carnobacterium*." The Prokaryotes. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt, Springer US: 320-403.

Hammes, W. P., Brandt, M. J., Francis, K. L., Rosenheim, J., Seitter, M. F. H. and Vogelmann, S. A. (2005). "Microbial ecology of cereal fermentations." Trends Food Sci Tech 16(1–3): 4-11.

Hammes, W. P. and Vogel, R. F. (1995). "The genus *Lactobacillus*." The Genera of Lactic Acid Bacteria. B. J. B. Wood and W. H. Holzapfel, Springer US. 2: 19-54.

Hersh, B. M., Farooq, F. T., Barstad, D. N., Blankenhorn, D. L. and Slonczewski, J. L. (1996). "A glutamate-dependent acid resistance gene in *Escherichia coli*." J Bacteriol 178(13): 3978-81.

Higuchi, T., Hayashi, H. and Abe, K. (1997). "Exchange of glutamate and gamma-aminobutyrate in a *Lactobacillus* strain." J Bacteriol 179(10): 3362-4.

Hutkins, R. W. and Nannen, N. L. (1993). "pH homeostasis in lactic acid bacteria." J Dairy Sci 76(8): 2354-2365.

Kaditzky, S. B., Behr, J., Stocker, A., Kaden, P., Gänzle, M. G. and Vogel, R. F. (2008). "Influence of pH on the formation of glucan by *Lactobacillus reuteri* TMW 1.106 exerting a protective function against extreme pH values." Food Biotechnol 22(4): 398-418.

Kandler, O. (1983). "Carbohydrate metabolism in lactic acid bacteria." Anton Van Leeuw J Microb 49(3): 209-24.

Katina, K. and Poutanen, K. (2013). "Nutritional aspects of cereal fermentation with lactic acid bacteria and yeast." Handbook on Sourdough Biotechnology. M. Gobbetti and M. Gänzle, Springer US: 229-244.

Kim, D. S., Thomas, S. and Fogler, H. S. (2000). "Effects of pH and trace minerals on long-term starvation of *Leuconostoc mesenteroides*." Appl Environ Microbiol 66(3): 976-81.

Konings, W. N. (2002). "The cell membrane and the struggle for life of lactic acid bacteria." Anton Van Leeuw J Microb 82(1-4): 3-27.

Kullin, B., Abratt, V. R. and Reid, S. J. (2006). "A functional analysis of the *Bifidobacterium longum cscA* and *scrP* genes in sucrose utilization." Appl Microbiol Biotechnol 72(5): 975-81.

Leroy, F. and De Vuyst, L. (2004). "Lactic acid bacteria as functional starter cultures for the food fermentation industry." Trends Food Sci Tech 15(2): 67-78.

Litwin, C. M. and Calderwood, S. B. (1993). "Role of iron in regulation of virulence genes." Clin Microbiol Rev 6(2): 137-49.

Lu, P., Ma, D., Chen, Y., Guo, Y., Chen, G. Q., Deng, H. and Shi, Y. (2013). "L-glutamine provides acid resistance for *Escherichia coli* through enzymatic release of ammonia." Cell Res 23(5): 635-44.

Ma, D., Lu, P. and Shi, Y. (2013). "Substrate selectivity of the acid-activated glutamate/gamma-aminobutyric acid (GABA) antiporter GadC from *Escherichia coli*." J Biol Chem 288(21): 15148-53.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D. M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J. H., Diaz-Muniz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B. and Mills, D. (2006). "Comparative genomics of the lactic acid bacteria." Proc Natl Acad Sci U S A 103(42): 15611-6.

Meroth, C. B., Walter, J., Hertel, C., Brandt, M. J. and Hammes, W. P. (2003). "Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis." Appl Environ Microbiol 69(1): 475-82.

Mobley, H. L., Island, M. D. and Hausinger, R. P. (1995). "Molecular biology of microbial ureases." Microbiol Rev 59(3): 451-80.

Molenaar, D., Bosscher, J. S., ten Brink, B., Driessen, A. J. and Konings, W. N. (1993). "Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in *Lactobacillus buchneri*." J Bacteriol 175(10): 2864-70.

Pereira, C. I., Matos, D., San Romao, M. V. and Crespo, M. T. (2009). "Dual role for the tyrosine decarboxylation pathway in *Enterococcus faecium* E17: response to an acid challenge and generation of a proton motive force." Appl Environ Microbiol 75(2): 345-52.

Reuter, G. (2001). "The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession." Curr Issues Intest Microbiol 2(2): 43-53.

Richard, H. and Foster, J. W. (2004). "*Escherichia coli* glutamate- and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential." J Bacteriol 186(18): 6032-41.

Rollan, G., Lorca, G. L. and Font de Valdez, G. (2003). "Arginine catabolism and acid tolerance response in *Lactobacillus reuteri* isolated from sourdough." Food Microbiol 20(3): 313-319.

Sanders, J. W., Leenhouts, K., Burghoorn, J., Brands, J. R., Venema, G. and Kok, J. (1998). "A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation." Mol Microbiol 27(2): 299-310.

Sanders, M. E. (2008). "Probiotics: definition, sources, selection, and uses." Clin Infect Dis 46(Supplement 2): S58-S61.

Schieberle, P. and Grosch, W. (1985). "Identification of the volatile flavour compounds of wheat bread crust — comparison with rye bread crust." Z Lebensm Unters Forsch 180(6): 474-478.

Schiffrin, E. J. and Blum, S. (2001). "Food processing: probiotic microorganisms for beneficial foods." Curr Opin Biotech 12(5): 499-502.

Schwab, C., Walter, J., Tannock, G. W., Vogel, R. F. and Gänzle, M. G. (2007). "Sucrose utilization and impact of sucrose on glycosyltransferase expression in *Lactobacillus reuteri*." Syst Appl Microbiol 30(6): 433-43.

Shalaby, A. R. (1996). "Significance of biogenic amines to food safety and human health." Food Res Int 29(7): 675-690.

Silla Santos, M. H. (1996). "Biogenic amines: their importance in foods." Int J Food Microbiol 29(2-3): 213-31.

Siragusa, S., De Angelis, M., Di Cagno, R., Rizzello, C. G., Coda, R. and Gobbetti, M. (2007). "Synthesis of gamma-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses." Appl Environ Microbiol 73(22): 7283-90.

Smits, H. H., Engering, A., van der Kleij, D., de Jong, E. C., Schipper, K., van Capel, T. M., Zaat, B. A., Yazdanbakhsh, M., Wierenga, E. A., van Kooyk, Y. and Kapsenberg, M. L. (2005). "Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin." J Allergy Clin Immunol 115(6): 1260-7.

Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., Coton, E., Coton, M., Barnavon, L., Bach, B., Rattray, F., Bunte, A., Magni, C., Ladero, V., Alvarez, M., Fernandez, M., Lopez, P., de Palencia, P. F., Corbi, A., Trip, H. and Lolkema, J. S. (2010). "Biogenic amines in fermented foods." Eur J Clin Nutr 64 Suppl 3: S95-100.

Stiles, M. E. and Holzapfel, W. H. (1997). "Lactic acid bacteria of foods and their current taxonomy." Int J Food Microbiol 36(1): 1-29.

Stolz, P., Böcker, G., Hammes, W. and Vogel, R. (1995a). "Utilization of electron acceptors by lactobacilli isolated from sourdough." Z Lebensm Unters Forsch 201(1): 91-96.

Stolz, P., Vogel, R. and Hammes, W. (1995b). "Utilization of electron acceptors by lactobacilli isolated from sourdough." Z Lebensm Unters Forsch 201(4): 402-410.

Stromeck, A., Hu, Y., Chen, L. and Gänzle, M. G. (2011). "Proteolysis and bioconversion of cereal proteins to glutamate and gamma-aminobutyrate (GABA) in rye malt sourdoughs." J Agric Food Chem 59(4): 1392-9.

Su, M., Schlicht, S. and Gänzle, M. (2011). "Contribution of glutamate decarboxylase in *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation." Microb Cell Fact 10(Suppl 1): S8.

Su, M. S., Oh, P. L., Walter, J. and Gänzle, M. G. (2012). "Intestinal origin of sourdough *Lactobacillus reuteri* isolates as revealed by phylogenetic, genetic, and physiological analysis." Appl Environ Microbiol 78(18): 6777-80.

Thiele, C., Gänzle, M. G. and Vogel, R. F. (2002). "Contribution of sourdough lactobacilli, yeast, and cereal enzymes to the generation of amino acids in dough relevant for bread flavor." Cereal Chem J 79(1): 45-51.

Titgemeyer, F. and Hillen, W. (2002). "Global control of sugar metabolism: a gram-positive solution." Anton Van Leeuw J Microb 82(1-4): 59-71.

Trindade, M. I., Abratt, V. R. and Reid, S. J. (2003). "Induction of sucrose utilization genes from *Bifidobacterium lactis* by sucrose and raffinose." Appl Environ Microbiol 69(1): 24-32.

Van Geel-Schutten, G. H., Faber, E. J., Smit, E., Bonting, K., Smith, M. R., Ten Brink, B., Kamerling, J. P., Vliegenthart, J. F. and Dijkhuizen, L. (1999). "Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains." Appl Environ Microbiol 65(7): 3008-14.

Vermeulen, N., Gänzle, M. G. and Vogel, R. F. (2007). "Glutamine deamidation by cereal-associated lactic acid bacteria." J Appl Microbiol 103(4): 1197-205.

Vogel, R. F., Knorr, R., Muller, M. R., Steudel, U., Gänzle, M. G. and Ehrmann, M. A. (1999). "Non-dairy lactic fermentations: the cereal world." Anton Van Leeuw J Microb 76(1-4): 403-11.

Walter, J. (2008). "Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research." Appl Environ Microbiol 74(16): 4985-96.

Walter, J., Britton, R. A. and Roos, S. (2011). "Host-microbial symbiosis in the vertebrate gastrointestinal tract and the *Lactobacillus reuteri* paradigm." Proc Natl Acad Sci U S A 108 Suppl 1: 4645-52.

Waterman, S. R. and Small, P. L. (1996). "Identification of sigma S-dependent genes associated with the stationary-phase acid-resistance phenotype of *Shigella flexneri*." Mol Microbiol 21(5): 925-40.

Weinberg, E. D. (1997). "The *Lactobacillus* anomaly: total iron abstinence." Perspect Biol Med 40(4): 578-83.

Wieser, H. (2007). "Chemistry of gluten proteins." Food Microbiol 24(2): 115-9.

Wilson, C. M., Leach, D., O'Toole, P. W. and Tannock, G. W. (2011). "Inactivation of the *Lactobacillus reuteri* 100-23 ureC gene affects in vitro acid tolerance, and impairs ecological fitness in vivo." Book of Abstracts, 10th Symposium on Lactic Acid Bacteria. Egmond aan Zee.

Wolken, W. A., Lucas, P. M., Lonvaud-Funel, A. and Lolkema, J. S. (2006). "The mechanism of the tyrosine transporter TyrP supports a proton motive tyrosine decarboxylation pathway in *Lactobacillus brevis.*" J Bacteriol 188(6): 2198-206.

CHAPTER TWO: **LEVANSUCRASE AND SUCROSE PHOSHORYLASE CONTRIBUTE TO RAFFINOSE, STACHYOSE, AND VERBASCOSE METABOLISM BY LACTOBACILLI**

A version of this chapter has been published in Food Microbiology.
Januana S. Teixeira, Victoria MacNeill, and Michael G. Gänzle.
(March 2012; 31:278-284)

CHAPTER 2

2.1. Introduction

Grain legumes of the *Fabaceae* family such as faba beans, field peas, and soybeans contain 2 – 10 % of α-galactosides of sucrose, namely raffinose (α-Gal-(1→6)-α-Glu-(1→2)-β-Fru), stachyose ([α-Gal-(1→6)]$_2$-α-Glu-(1→2)-β-Fru) and verbascose ([α-Gal-(1→6)]$_3$-α-Glu-(1→2)-β-Fru) (Guillon and Champ, 2002). These α-galactosides, also referred to as soybean oligosaccharides or raffinose family oligosaccharides (RFOs), are not degraded in the human upper gastrointestinal tract due to the absence of the α-galactosidase (α-Gal) (Gitzelmann and Auricchio, 1965). RFOs are fermented in the large intestine by the intestinal microbiota, causing gastrointestinal symptoms, including abdominal discomfort, flatulence, and diarrhea (Rackis *et al.*, 1970). These negative effects are dose dependent (Oku and Nakamura, 2002). In moderate doses, intestinal fermentation of RFOs favors metabolism of beneficial intestinal organisms such as bifidobacteria. Therefore, RFOs have also been referred to as prebiotics (reviewed by Van Loo *et al.*, 1999). Dose dependent beneficial or adverse effects of RFOs match observations for other non-digestible oligosaccharides, and suggest the necessity of careful control of oligosaccharides levels in food (Oku and Nakamura, 2002).

The concentration of RFOs in legumes of the *Fabaceae* family has been reduced by treatment with α-Gal (EC 3.2.1.22) or with microorganisms capable of degrading RFOs. Dietary supplementation of α-Gal (Beano®) prevents flatus after consumption of pulses (Levine and Weisman, 2004). Many lactic acid bacteria (LAB), including food fermenting lactobacilli and *Leuconostoc* species, produce α-Gal, and have been used to eliminate RFOs in food prepared from soy, pinto

beans, cowpea or field pea flours, or pearl millet (Mital *et al.*, 1973; Doblado *et al.*, 2003; Duskiewicz-Reinhard *et al.*, 2006; Songre-Ouattara *et al.*, 2008; Yoon and Hwang, 2008). Selection of LAB to remove RFOs in legumes has to date focused only on their α-Gal activity (for review see Savoy de Giori *et al.*, 2010). However, partial hydrolysis of raffinose can be achieved by fructansucrases, such as levansucrase (EC 2.4.1.10), a glycoside hydrolase enzyme present in many lactobacilli (Tieking *et al.*, 2003). Levansucrase cleaves the glycosidic bond between the glucose and fructose in sucrose and raffinose (reviewed by van Hijum *et al.*, 2006). However, a contribution of levansucrase to raffinose metabolism in food has not been investigated, and it remains unknown whether levansucrase also degrades stachyose and verbascose.

It was the aim of this study to investigate the role of levansucrase and sucrose phorphorylase in RFO metabolism to achieve their partial reduction without complete elimination. Metabolism of raffinose, stachyose and verbascose was analyzed with the strains *Lactobacillus reuteri* LTH5448 and *Lactobacillus sanfranciscensis* LTH2590. *L. reuteri* LTH5448 expresses a levansucrase FtfA and an intracellular α-Gal; *L. sanfranciscensis* LTH2590 expresses the levansucrase LevS but has no α-Gal activity (Tieking *et al.*, 2005; Schwab *et al.*, 2007). Growth and metabolite formation of parent strains and the isogenic levansucrase-negative *L. reuteri* LTH5448 *ftfA* mutant and *L. sanfranciscensis* LTH2590 *levS* mutant, were compared. Experiments were performed in laboratory media and in a food model system, pulse sourdoughs for use in gluten-free baking.

2.2. Materials and methods

2.2.1. Strains, media and growth conditions

Two pairs of strains used in this experiment were chosen based on their levansucrase and α-Gal genes activities as shown in Table 2-1. The organisms were anaerobically cultivated at 37°C or 30°C in modified de Man-Rogosa-Sharpe medium containing maltose, glucose, and fructose (mMRS) (Stolz *et al.*, 1995); or modified MRS containing 20 g/L glucose (gluMRS); 20 g/L sucrose (sucMRS); or 20 g/L raffinose (rafMRS) as sole carbon sources. The levansucrase deletion mutants *L. reuteri* LTH5448 *ftfA* and *L. sanfranciscensis* LTH2590 *levS* were grown in presence of erythromycin (5 mg/L in liquid medium, 10 mg/L in agar plates). For preparation of working cultures, strains were obtained from -80°C stock cultures, streaked on mMRS plates, and subcultured in mMRS prior to use.

Table 2-1. Strains and properties used in the study.

Strain	α-Gal	Levansucrase	Incubation Temp (°C)
Lactobacillus reuteri LTH5448	+	+	37
L. reuteri LTH5448 *ftfA* mutant (Schwab et al., 2007)	+	-	37
Lactobacillus sanfranciscensis LTH2590	-	+	30
L. sanfranciscensis LTH2590 *levS* mutant (Tieking et al., 2005)	-	-	30

2.2.2. General molecular technique

Genomic DNA was isolated from overnight cultures grown in mMRS using DNeasy Blood & Tissue kit (Qiagen, Mississauga, Canada) according to the instructions of the manufacturer. DNA was amplified by PCR with Taq DNA polymerase and dNTPs from Invitrogen (Burlington, Canada). Primers were purchased from Integrated DNA technologies (Coralville, USA).

2.2.3. Isolation of mRNA and synthesis of cDNA libraries

RNA was isolated from 1 mL exponentially growing cultures (OD_{600} of 0.5). Two volumes of RNAprotect (Qiagen) were added, and cells were harvested by centrifugation at 5000 × *g*. Cells were resuspended in 1 mL TRIzol (Invitrogen), and disrupted using a MiniBeadBeater-8 (Biospec products, Bartlesville, USA) and 0.1 mm zirconia-silica beads. The supernatant was transferred to 1.5 mL Eppendorf tubes for RNA isolation according to TRIzol manufacture's manual. Reagents and enzymes for DNase treatment and reverse transcription were purchased from Promega (Madison, USA) and used according to the manufacturer's instructions.

2.2.4. Analysis of transcriptional levels of the **scrP** *gene in response to sucrose, glucose, and raffinose*

Quantitative analysis of the expression of sucrose phosphorylase gene (*scrP*) in *L. reuteri* and *L. reuteri ftfA* mutant was carried out using cDNA as a template in Reverse-Transcriptase-quantitative-PCR (RT-qPCR) reactions. DNase

treated mRNA preparations were used as negative control and chromosomal DNA were used as positive control. The following primers were used for specific gene *scrP*: Sucpho-F (5'-CTGGTAAGAACCGTCCSAAC-3') and Sucpho-R (5'-CAGTTAAGATATCCTTAGCATC-3'); and reference gene phosphoketolase (*pho*): Phoket-F (5'-GTAACCTTCAAGGAATCC-3') and Phoket-R (5'-CGTCTTTACGCATTCCTTG-3') (Schwab *et al.*, 2007). Amplifications were carried out in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Melting curve analysis and determination of amplicon size by agarose gel electrophoresis verified amplification of the appropriate transcripts. Expression levels of sucrose phosphorylase in cultures grown in sucMRS or rafMRS (N) were calculated according to Pfaffl (2001), using the phosphoketolase gene *pho* as housekeeping gene and cultures grown in gluMRS as reference conditions (N_0). The efficiencies of *sucP* and *pho* PCR reactions were determined as 2. Data are reported as $\log(N/N_0)$. Gene expression was quantified in triplicate independent experiments and each experiment was analysed in duplicate.

2.2.5. Determination of growth kinetics and metabolite formation in mMRS media

Growth of *L. reuteri* and *L. reuteri ftfA* mutant was assessed in mMRS or media containing glucose (gluMRS), sucrose (sucMRS), or raffinose (rafMRS) as sole carbon source. Media were inoculated with overnight culture, and growth was monitored by changes in the optical density (OD) of the cultures at 600 nm

over 24 hours. Samples were taken for HPLC as described below. Data shown are representative for three independent experiments. Significance was determined by student's t-test using SigmaPlot software (Systat Software, Inc., San Jose, CA) and $p < 0.05$.

2.2.6. Determination of growth kinetics and metabolite formation in pulse doughs

Faba bean (*Vicia faba*) and field pea (*Pisum sativum*) flours were used for the sourdough fermentation with *L. reuteri* and *L. sanfranciscensis*, and their respective levansucrase negative mutants. Overnight cultures were washed in peptone buffer (phosphate buffered saline: 137 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), and resuspended in the same volume of sterilized tap water. Ten grams of faba bean or field pea flours were mixed with 10 mL of sterilized tap water and 0.1 mL of starter culture. Doughs were incubated at the respective ideal temperatures, and samples were taken over 24 hours for pH, cell counts, and HPLC analysis of carbohydrate metabolism and metabolite formation. Sample preparation for HPLC was done as described below. Data shown are representative for two independent experiments.

2.2.7. Sourdough fermentation and bread baking

Strains from overnight cultures were inoculated to the dough to obtain a concentration of 10^7 CFU/g in the final sourdough. The bread formula was based on sorghum sourdough (Schwab *et al.*, 2008). Bread dough was prepared with 30

g of faba bean flour, 30 g of sorghum flour (Bob's Red Mill, Milwaukie, USA), 130 g of white rice flour (Bob's Red Mill), 20 g of cornstarch (Canada Cornstarch, Memphis, USA), 2 g of salt, 2 g of sugar, and 2 g of baker's yeast with 140 mL of tap water (Table 2-2). The ingredients were all added at the same time and mixed for 3 minutes in a 300 W Kitchen aid mixer with a spiral kneader. The dough was placed in the bread trays and proofed at 37°C with 80% relative humidity for 2 hours. Samples were taken after kneading, and after proofing for HPLC analysis of sugars and metabolites. Bread was baked at 200°C for 40 minutes. In addition to the straight dough process used as control, bread dough was prepared from unfermented bean flour sorghum sourdough, or with fermented bean flour and sorghum sourdough (Table 2-2). Samples from the fermentation and dough were taken over 24 hours for pH, cell counts, and HPLC analysis of metabolites and sugars. Sample preparation for HPLC was done as described below. Analysis of bread doughs was carried out in two independent experiments.

Table 2-2. Ingredients for sourdough bread baking.

Ingredients [g]	Fermented bean flour, sorghum sourdough	Bean flour, sorghum sourdough	Control
Water	110	110	140
Faba bean flour	0	30	30
Fermented faba bean flour[a]	30	0	0
Sorghum flour	0	0	30
Sorghum sourdough[a]	60	60	0
White rice flour	130	130	130
Cornstarch	20	20	20
Salt	2	2	2
Sugar	2	2	2
Yeast	2	2	2

[a)] Faba bean and sorghum flours were fermented with *L. reuteri* LTH5448 for 24 hours at 37°C.

2.2.8. Quantification of oligosaccharides, monosaccharides, and microbial metabolites

Glucose, fructose, melibiose, sucrose, maltose, raffinose, stachyose, and verbascose were analyzed by high performance anion exchange chromatography coupled to pulsed amperometric detection (HPAEC-IPAD). All standards were obtained from Sigma (Oakville, Canada). Sugars were separated using a CarbopacPA20 column (Dionex, Oakville, Canada) with water (A), 0.2 M NaOH (B), and 1 M NaAcO (C) as solvents at a flow rate of 0.25 mL/min with the following gradients over time: t=0, 30.4% B, 1.3% C; t=22 min, 30.4% B, and 11.34% C, followed by washing and regeneration. For analysis of carbohydrates in mMRS, cells were removed by centrifugation (5000 × *g*, 4°C, 10 min) and the supernatants were diluted 100 times with deionized water. Flour and dough samples were extracted with water at an extraction ratio of 1:10 (w/v) at 80°C for 2 hours, and centrifuged (12000 × *g*, 4°C, 10 min) prior dilution of supernatants.

Organic acids, ethanol and mannitol were determined using an Aminex HPX-87 column (Bio-Rad, Mississauga, Canada) at a temperature of 70°C, using 5 mM H_2SO_4 as solvent, at a flow rate of 0.4 mL/min. A refractive index detector was used for visualization. The concentrations of lactate, acetate, ethanol, and mannitol were determined using external standards. For analysis of from mMRS sample, cells were removed by centrifugation (5000 × *g*, 4°C, 10 min) and the supernatants were diluted with an equal volume of 7% perchloric acid and incubated overnight at 4°C. Precipitates were removed by centrifugation (10000 × *g*, 4°C, 10 min). The samples from flour and dough were extracted with water at

an extraction ratio of 1:6 (w/v) at 80°C for 2 hours and centrifuged (12000 × *g*, 4°C, 10 min) prior dilution of supernatants.

2.3. Results

2.3.1. Contribution of levansucrase to raffinose metabolism

To determine a contribution of levansucrase to raffinose metabolism, growth and metabolism of *L. reuteri* and its *ftfA* mutant were compared in maltose, glucose and fructose (mMRS), glucose (gluMRS), sucrose (sucMRS), or raffinose (rafMRS) as substrate (Figure 2-1). In mMRS and sucMRS, wild type and mutant showed faster growth compared with the growth in gluMRS and rafMRS. The wild type strain but not the *ftfA* mutant strain accumulated melibiose as a metabolic intermediate from raffinose, indicating extracellular metabolism by levansucrase (Figure 2-2). In presence of sucrose, the *ftfA* mutant strain did not produce 1-kestose and fructooligosaccharides (FOS). After 24 hours of growth both strains completely metabolized all sugars in all four media (data not shown).

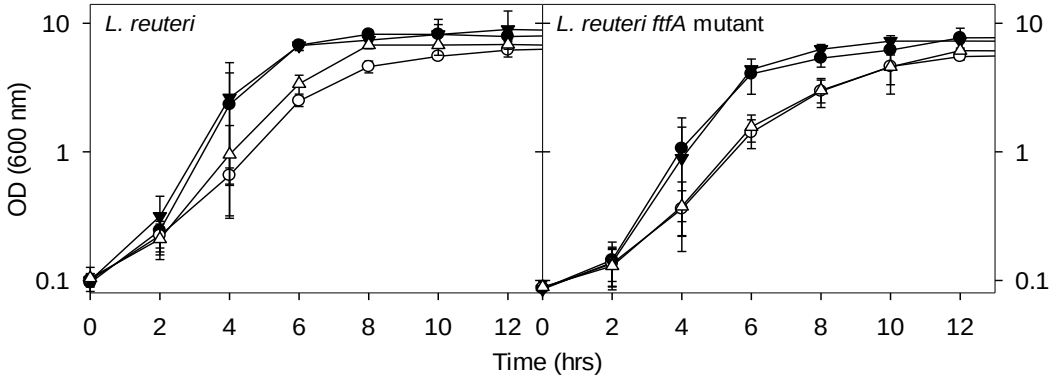


Figure 2-1. Growth kinetics of *L. reuteri* LTH5448 and *ftfA* mutant in mMRS (●), gluMRS (○), sucMRS (▼), and rafMRS (Δ). Symbols indicate means ± standard deviation from triplicate independent experiments.

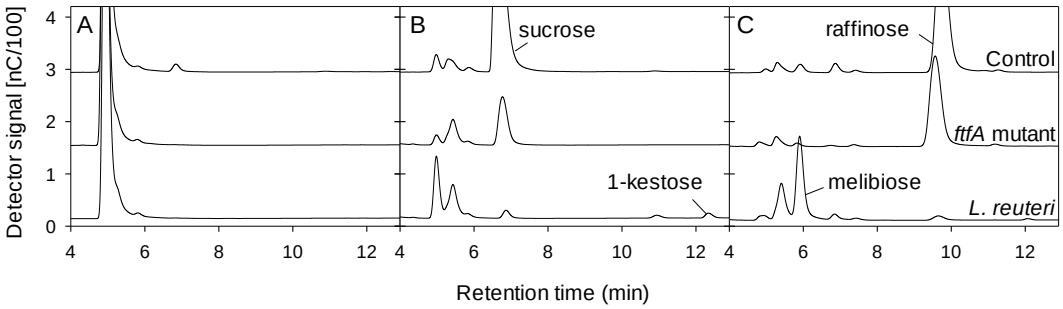


Figure 2-2. Separation of culture supernatant by HPAEC-IPAD after 8 hours of growth of *L. reuteri* LTH5448 and its *ftfA* mutant at 37°C. Strains were grown in gluMRS (A), sucMRS (B), and rafMRS (C); unfermented medium served as control. Chromatograms are offset by 100 nC. The identity of peaks is indicated. After 24 hours, *L. reuteri* LTH5448 as well as the *ftfA* mutant completely metabolized all sugars in the media (data not shown). Data are representative of three independent experiments.

L. reuteri and its *ftfA* mutant formed comparable amounts of lactate and ethanol. Sucrose or raffinose supported mannitol and acetate formation by both strains (Table 2-3). Ethanol formation was lower, and mannitol and acetate formation was higher in sucMRS than in rafMRS. Additionally, the *L. reuteri ftfA* mutant strain produced higher levels of mannitol and acetate in sucMRS compared to the wild type strain.

Table 2-3. Metabolite formation by *L. reuteri* LTH5448 and its *ftfA* mutant after growth in glucose (gluMRS), sucrose (sucMRS), or raffinose (rafMRS) as substrate for 24 h. Means ± standard deviations of experiments performed in triplicate are shown.

Medium	***L. reuteri***	**Metabolites [mmol / L]**			
		Mannitol	**Lactate**	**Acetate**	**Ethanol**
gluMRS	LTH5448	0 ± 0	62 ± 1	2 ± 0	106 ± 7
	ftfA mutant	0 ± 0	69 ± 6	2 ± 0	112 ± 6
sucMRS	LTH5448	28 ± 1	31 ± 0	13 ± 0	21 ± 5
	ftfA mutant	34 ± 1[a]	32 ± 2	16 ± 1[a]	22 ± 9
rafMRS	LTH5448	15 ± 3	39 ± 6	8 ± 1	47 ± 9
	ftfA mutant	18 ± 2	42 ± 7	9 ± 1	53 ± 10

[a)] designates significant differences in metabolite concentrations in the same growth medium between *L. reuteri* LTH5448 and the *ftfA* mutant ($p < 0.05$).

2.3.2. Contribution of sucrose phosphorylase expression to raffinose metabolism

Expression of sucrose phosphorylase (ScrP, EC 2.4.1.7) in *L. reuteri* LTH5448, the alternative pathway for sucrose utilization, is induced in presence of sucrose (Schwab *et al.*, 2007). In the *ftfA* mutant of *L. reuteri* LTH5448, ScrP is the only enzyme capable of sucrose metabolism (Schwab *et al.*, 2007). Sucrose is released from raffinose, stachyose and verbascose after hydrolysis by intracellular α-Gal. The expression of *scrP* in *L. reuteri* and its *ftfA* mutant in response to addition of sucrose and raffinose to the growth substrate was quantified relatively to its expression in gluMRS. Both *L. reuteri* and its *ftfA* mutant over-expressed *scrP* in presence of sucrose or raffinose. The $\log_{10}$ expression ratios in presence of sucrose were 2.76 ± 0.6 and 2.49 ± 0.39 for *L. reuteri* and *ftfA* mutant, respectively; the expression ratios of *scrP* in presence of raffinose were 2.95 ± 0.36 for *L. reuteri* LTH5448 and 2.71 ± 0.32 for its *ftfA* mutant. The induction of sucrose phosphorylase expression by raffinose indicates a contribution of sucrose phosphorylase to raffinose metabolism.

2.3.3. Growth kinetics and carbohydrate metabolism in pulse sourdoughs

To elucidate the relevance of levansucrase for stachyose and verbascose degradation in pulse sourdoughs, the growth and metabolism of *L. reuteri*, *L. sanfranciscensis*, and their respective levansucrase deficient mutants were investigated in faba bean and field pea fermentations. In both legume flours, *L. reuteri* and its *ftfA* mutant grew rapidly as represented by the decrease in pH

after 8 hours (Figure 2-3). *L. sanfranciscensis* grew only after 24 hours; its *levS* mutant did not grow (Figure 2-3) and cell counts remained 10^7 CFU/g or below throughout fermentation.

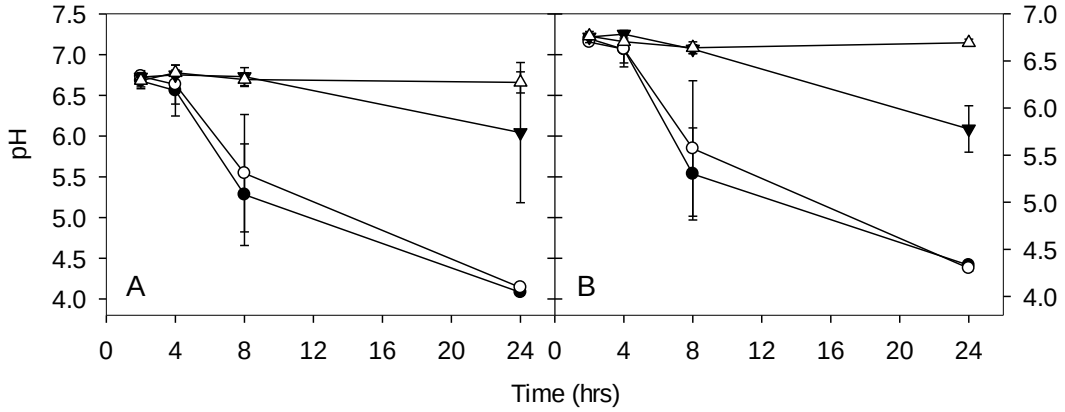


Figure 2-3. Development of pH values for *L. reuteri* LTH5448 (●), *ftfA* mutant (○), *L. sanfranciscensis* LTH2590 (▼), and *levS* mutant (Δ) during growth in field pea sourdoughs (Panel A) and faba bean sourdoughs (Panel B). Symbols indicate means ± standard deviation from duplicate independent experiments.

Carbohydrate concentrations in faba bean fermentations are shown in Figure 2-4. The concentrations of sucrose, raffinose, stachyose and verbascose decreased rapidly in fermentations with *L. reuteri*. The concentration of melibiose increased to about 1 mmol / kg at 8 h and decreased after that. In addition, two other peaks were observed in fermentations with levansucrase positive strains. These peaks increased and decreased concomitantly with melibiose concentrations in *L. reuteri* LTH5448, and increased concomitantly with melibiose in *L. sanfranciscensis* LTH2590. Based on the retention time, their formation by levansucrase, and the use of a manninotriose external standard, these peaks were identified as manninotriose (6'(Gal)-melibiose), and manninotetraose

(6'6'$(Gal)_2$-melibiose). The *L. reuteri ftfA* mutant metabolized sucrose, raffinose, stachyose and verbascose, but the α-GOSs melibiose, manninotriose or manninotetraose were not formed. *L. sanfranciscensis* LTH2590 metabolised raffinose, stachyose and verbascose, and accumulated about 1 mmol/kg melibiose as well as manninotriose and manninotetraose. These α-GOSs were not degraded due to the lack of α-Gal activity. In the *L. sanfranciscensis levS* mutant, all sugars decreased slightly over the fermentations time and formation of α-GOSs was not observed. Qualitatively comparable data were observed in fermentations with field pea flour performed in duplicate (data not shown).

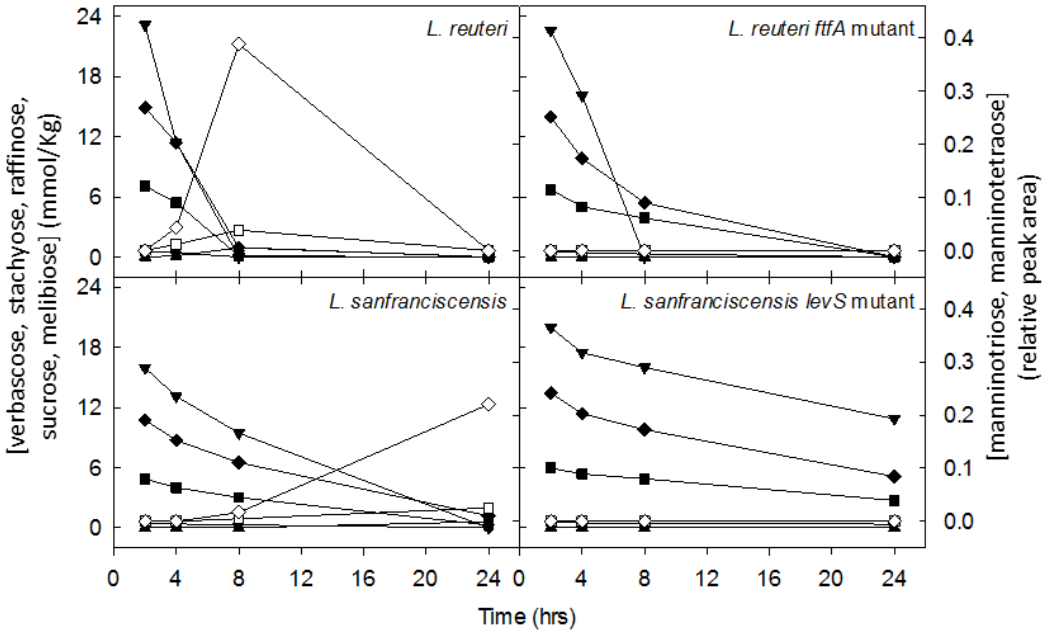


Figure 2-4. Carbohydrate concentrations in faba bean fermentations with *L. reuteri* LTH5448, *L. sanfranciscensis* LTH2590 and their isogenic levansucrase-deficient mutants. Symbols indicate concentrations of sucrose (▼), melibiose (▲), raffinose (●), stachyose (■), verbascose (♦); relative peak area are shown for the α-GOS manninotriose (□), manninotetraose (◊). Data are qualitatively representative for two independent experiments with faba bean flour.

The results of organic acid formation were consistent with the consumption of carbohydrates. *L. reuteri* LTH5448 produced large amounts of lactate and comparable amounts of acetate and ethanol, while the *ftfA* mutant produced less lactate, acetate, and ethanol (data not shown). *L. sanfranciscensis* LTH2590 produced lactate, acetate and ethanol but its *levS* did not accumulate any of the metabolites.

2.3.4. Quantification of oligosaccharides in preparation of gluten-free breads

Fermentation of pulse flours indicated that levansucrase is responsible for rapid conversion of RFOs to α-GOSs, while intracellular metabolism by α-Gal is slower. In order to investigate whether fermentation with levansucrase-positive lactobacilli can be used to control the level of oligosaccharides in gluten-free baking, the utilization of carbohydrates and the formation of metabolites in bread doughs prepared from bean, sorghum, and white rice flours were analyzed. The process included the use of fermented pulse flours to achieve complete metabolism of RFOs, or a combination of sorghum sourdough and unfermented bean flours to achieve high cell counts of lactobacilli for rapid conversion of RFOs to α-GOSs. The concentration of sugars is shown in Table 2-4. The metabolite concentrations, pH and cell counts for the bread fermentation match results from carbohydrate metabolism (data not shown). Overnight fermentation of bean flour resulted in complete degradation of RFOs; however, when sorghum sourdough was used in combination with unfermented bean flour, RFOs were only partially degraded after kneading and proofing and the α-GOSs melibiose, manninotriose and manninotetraose were detected (Table 2-4 and Figure 2-5).

Remarkably, RFO conversion to α-GOSs in control doughs was comparable to bread dough prepared from sorghum sourdough and unfermented bean flour. Because the control dough did not contain (levansucrase-positive) lactobacilli, this conversion may be attributable to the yeast invertase activity. The ethanol concentration for all trials was high which due to yeast activity in the bread dough. Ethanol formation was lowest, and lactate and acetate formation was highest in dough prepared from fermented bean flour and sorghum sourdough (data not shown).

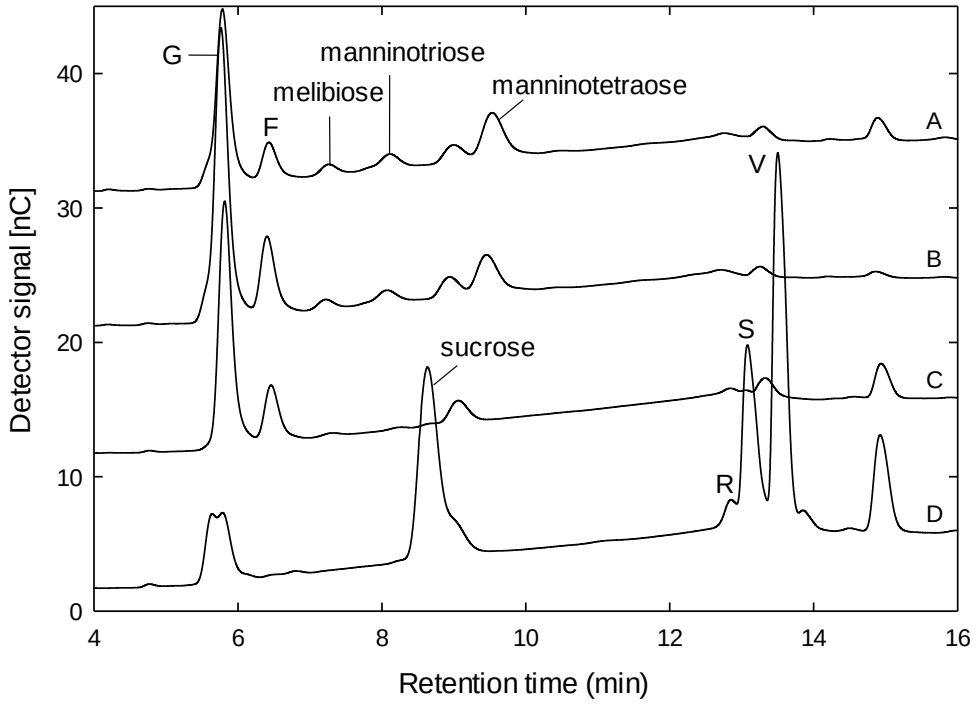


Figure 2-5. Separation of mono- and oligosaccharides from selected bread doughs after proofing. Chromatograms are offset by 10 nC. The identity of peaks is indicated; G, glucose and galactose; F, fructose; R, raffinose; S, stachyose; V, verbascose. Doughs were prepared from unfermented ingredients (control, trace A) or unfermented bean flour and sorghum sourdough (trace B). The oligosaccharide composition of sorghum sourdough (trace C) and bean flour (trace D) prior to fermentation is additionally shown for comparison. Data are representative for three independent experiments.

Table 2-4. Concentration of oligosaccharides in bread dough fermented with baker's yeast and *L. reuteri* LTH5448. Doughs were prepared with sorghum sourdough and fermented bean flour, with sorghum sourdough and unfermented bean flour, or unfermented bean and sorghum flours (Control). Means ± standard deviations of experiments performed in duplicate are shown.

	Fermented bean flour, sorghum sourdough		**Bean flour, sorghum sourdough**		**Control**	
[Substrates] [Metabolites] [mmol/Kg]	**After Kneading**	**After Proofing**	**After Kneading**	**After Proofing**	**After Kneading**	**After Proofing**
Sucrose	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Raffinose	0 ± 0	0 ± 0	0.4 ± 0.6	0.1 ± 0	0.2 ± 0	0.1 ± 0
Stachyose	0 ± 0	0 ± 0	0.6 ± 0	0 ± 0	0 ± 0	0 ± 0
Verbascose	0 ± 0	0 ± 0	1 ± 0.6	0 ± 0	0.5 ± 0.7	0 ± 0
Melibiose	0 ± 0	0 ± 0	0.2 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.5 ± 0.1

2.4. Discussion

This study investigated the role of levansucrase in RFO metabolism of lactobacilli, and evaluated whether levansucrase-positive lactobacilli can be employed for conversion of RFOs to α-GOSs in fermentation of grain legumes of the *Fabaceae* family. The function of levansucrase gene was investigated by comparing RFO metabolism in *L. reuteri* and *L. sanfranciscensis* and two isogenic, levansucrase-negative mutants (Tieking *et al.*, 2005; Schwab *et al.*, 2007).

Degradation of raffinose by LAB is achieved by the action of α-Gal, an intracellular enzyme that degrades raffinose to galactose and sucrose as the final product of hydrolysis (Yoon and Hwang, 2008). In bifidobacteria, sucrose and raffinose induce sucrose phosphorylase (ScrP) activity, an intracellular enzyme that catalyzes phosphorolysis of sucrose (Trindade *et al.*, 2003). ScrP has a

specific phosphorolytic activity for the conversion of sucrose and inorganic phosphate into α-D-glucose 1- phosphate and fructose but is not active with raffinose as substrate. During catalysis, ScrP covalently binds the glucose moiety of sucrose (Luley-Goedl and Nidetzky, 2010); binding is sterically hindered for α-galactosides of sucrose. In lactobacilli expressing α-Gal and ScrP, metabolism of RFOs thus proceeds via transport, α-Gal activity to release sucrose from RFOs, and sucrose phosphorolysis by ScrP (Figure 2-6), analogous to RFO metabolism by bifidobacteria (Kullin *et al.*, 2006). The induction of *scrP* in *L. reuteri* was previously reported (Schwab *et al.*, 2007); in agreement with studies in bifidobacteria (Trindade *et al.*, 2003), *scrP* in *L. reuteri* was also induced by raffinose.

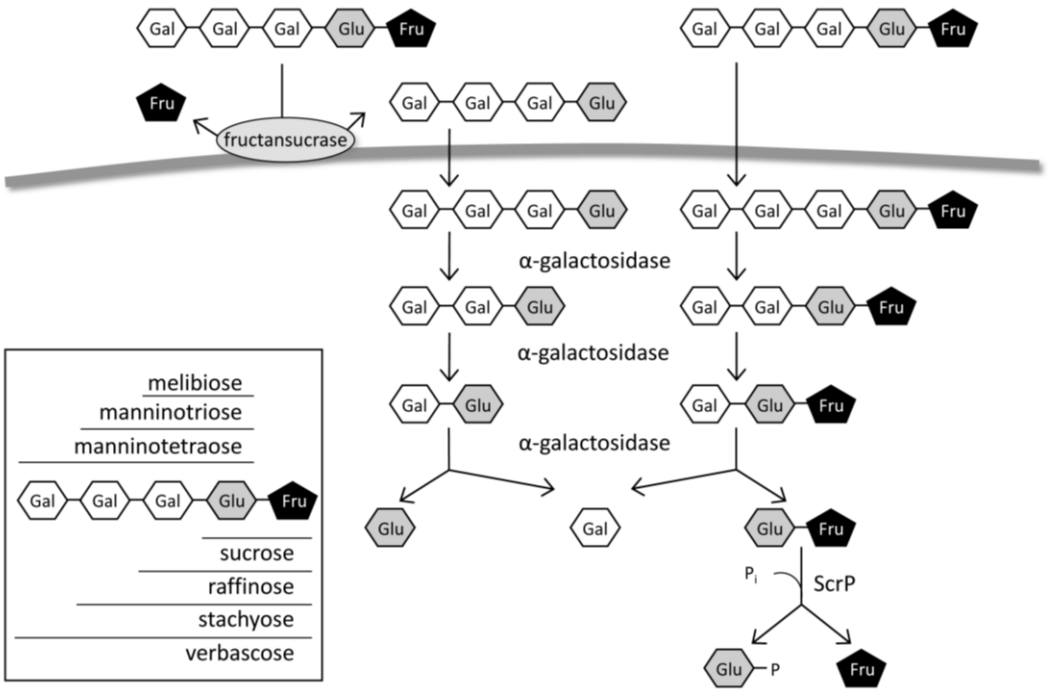


Figure 2-6. Metabolic pathways of verbascose utilization in *L. reuteri* LTH5448. Corresponding pathways result in degradation of stachyose and raffinose.

Levansucrases cleave the bond between fructose and glucose in sucrose and raffinose to yield glucose and melibiose, respectively (van Hijum *et al.*, 2006). In lactobacilli expressing levansucrase and α-Gal, extracellular conversion of RFOs to α-GOSs, followed by α-GOSs transport and intracellular hydrolysis thus provides an alternative pathway for RFO metabolism (Figure 2-6). Levansucrases bind the fructose moiety of sucrose or raffinose; analysis of the levansucrase structure demonstrated that the fructose- and glucose moieties but not the galactose moiety of raffinose are associated with the catalytic site of levansucrase (Seibel *et al.*, 2006). Binding of stachyose and verbascose to the catalytic center of levansucrase is thus not sterically hindered. By comparison of RFO metabolism by two isogenic pairs of wild type strains and levansucrase-deficient mutants; this study demonstrated that stachyose and verbascose are indeed substrates for bacterial levansucrases.

The two pathways for RFO metabolism coexist in *L. reuteri* LTH5448 but the preferential use of the levansucrase / α-Gal pathway may be explained by the location of hydrolytic enzymes (Figure 2-6). Metabolism by α-Gal / ScrP requires transport of RFOs prior to hydrolysis by intracellular enzymes. Metabolism by levansucrase / α-Gal allows for extracellular conversion of RFOs, followed by the uptake of α-GOSs with a lower degree of polymerisation. The accumulation of melibiose, manninotriose and manninotetraose by *L. reuteri* LTH5448 indicates that RFO metabolism is limited by transport of oligosaccharides. The *L. reuteri ftfA* mutant utilized RFOs via α-Gal /ScrP pathway; however, metabolism was slower when compared to the wild type strain. In *L. sanfranciscensis* LTH2590,

levansucrase was the only enzyme available for RFO metabolism and α-GOSs were not degraded. Transport systems for RFOs were not yet identified in lactobacilli but the transport system for lactose and β-GOSs, LacS, also transports α-GOSs (Poolman *et al.*, 1992).

The distinctions in organic acid production between *L. reuteri* and its mutant confirm the difference in the efficiency of their RFO metabolism. *L. reuteri* converts fructose to mannitol via a NAD(P)H dependent mannitol dehydrogenase (Gänzle *et al.*, 2007).The lower level of mannitol and acetate production by *L. reuteri* LTH5448 when compared to its *ftfA* mutant indicates that the majority of fructose moieties from sucrose were used for FOSs and levan synthesis by levansucrase activity. The *ftfA* mutant strain did not produce 1-kestose and FOSs, but formed higher concentrations of mannitol and acetate, consistent with earlier observations (Schwab *et al.*, 2007).

The formulation of gluten-free bread recipes typically results in products with low amount of micronutrients and dietary fiber. The incorporation of legume flours in these recipes may increase the content of fiber and micronutrients in gluten-free breads (Gallagher *et al.*, 2004). However, the high levels of oligosaccharides in legumes are considered anti-nutritive factors, and lactic fermentation can be employed to improve their palatability and digestibility. The oligosaccharide analysis of gluten-free doughs indicated fast conversion of RFOs to α-GOSs by levansucrase. Interestingly, the control trial showed comparable results with bean flour and sorghum sourdough, which indicates that yeast

invertase, which has a catalytic mechanism comparable to levansucrase (Lammens *et al.*, 2009), also catalysed RFO conversion to α-GOSs.

The question whether RFOs are anti-nutritive factors or functional ingredients stimulating growth of beneficial intestinal bacteria is entirely dependent on their dose (Van Loo *et al.*, 1999; Oku and Nakamura, 2002). Oku and Nakamura (2002) estimated that intake of 0.3 g / kg body weight /day of non-digestible oligosaccharides is tolerated without adverse side effects. The present study provides insight into the conversion of RFOs to α-GOSs by levansucrase-positive fermentation. Levansucrase rapidly converts raffinose, stachyose, and verbascose to α-GOSs. This conversion reduces the load of non-digestible oligosaccharide by 33% (raffinose) to 20% (stachyose). Reduction of the oligosaccharide load may result in conversion of RFOs from "antinutritive factors" to "functional ingredients" that are tolerated without those adverse side effects that are typically associated with consumption of grain legumes. Since the α-galactosidic linkages are maintained, RFOs prebiotic activity in the body is also conserved. The use of *L. reuteri* for conversion of RFOs to α-GOS in gluten free bread dough provides proof of concept for the use of the fermentative RFO conversion in food product development.

2.5. References

Doblado, R., Frias, J., Munoz, R. and Vidal-Valverde, C. (2003). "Fermentation of Vigna sinensis var. carilla flours by natural microflora and *Lactobacillus* species." J Food Prot 66(12): 2313-20.

Duskiewicz-Reinhard, W., Gujska, E. and Khan, K. (2006). "Reduction of stachyose in legume flours by lactic acid bacteria." J Food Sci 59(1): 115-117.

Gallagher, E., Gormley, T. and Arendt, E. (2004). "Recent advances in the formulation of gluten-free cereal-based products." Trends Food Sci Technol 15(3-4): 143-152.

Gänzle, M. G., Vermeulen, N. and Vogel, R. F. (2007). "Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough." Food Microbiol 24(2): 128-38.

Gitzelmann, R. and Auricchio, S. (1965). "The Handling of Soya Alpha-Galactosides by a Normal and a Galactosemic Child." Pediatrics 36: 231-5.

Guillon, F. and Champ, M. M. (2002). "Carbohydrate fractions of legumes: uses in human nutrition and potential for health." Br J Nutr 88 Suppl 3: S293-306.

Kullin, B., Abratt, V. R. and Reid, S. J. (2006). "A functional analysis of the *Bifidobacterium longum* cscA and scrP genes in sucrose utilization." Appl Microbiol Biotechnol 72(5): 975-81.

Lammens, W., Le Roy, K., Schroeven, L., Van Laere, A., Rabijns, A. and Van den Ende, W. (2009). "Structural insights into glycoside hydrolase family 32 and 68 enzymes: functional implications." J Exp Bot 60(3): 727-40.

Levine, B. and Weisman, S. (2004). "Enzyme replacement as an effective treatment for the common symptoms of complex carbohydrate intolerance." Nutr Clin Care 7(2): 75-81.

Luley-Goedl, C. and Nidetzky, B. (2010). "Small-molecule glucosylation by sucrose phosphorylase: structure-activity relationships for acceptor substrates revisited." Carbohydr Res 345(10): 1492-6.

Mital, B. K., Shallenberger, R. S. and Steinkraus, K. H. (1973). "Alpha-glactosidase activity of lactobacilli." Appl Microbiol 26(5): 783-8.

Oku, T. and Nakamura, S. (2002). "Digestion, absorption, fermentation, and metabolism of funcional sugar substitutes and their available energy." Pure Applied Chemistry 74(7): 1253-1261.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res 29(9): e45.

Poolman, B., Modderman, R. and Reizer, J. (1992). "Lactose transport system of *Streptococcus thermophilus*. The role of histidine residues." J Biol Chem 267(13): 9150-7.

Rackis, J. J., Honig, D. H., Sessa, D. J. and Steggerda, F. R. (1970). "Flavor and flatulence factors in soybean protein products." J Agric Food Chem 18(6): 977-82.

Savoy de Giori, G., Agiorre, L., Marazza, J. and Garro, M. S. (2010). "An overview of lactic acid bacteria applications for healthful soy foods development." Biotechnology of lactic acid bacteria: novel applications. R. R. R. F. Mozzi, & G..M. Vignolo Ames, Iowa, Wiley-Blackwell.

Schwab, C., Mastrangelo, M., Corsetti, A. and Gänzle, M. (2008). "Formation of oligosaccharides and polysaccharides by *Lactobacillus reuteri* LTH5448 and *Weissella cibaria* 10M in sorghum sourdoughs." Cereal Chemistry 85(5): 679-684.

Schwab, C., Walter, J., Tannock, G. W., Vogel, R. F. and Gänzle, M. G. (2007). "Sucrose utilization and impact of sucrose on glycosyltransferase expression in *Lactobacillus reuteri*." Syst Appl Microbiol 30(6): 433-43.

Seibel, J., Moraru, R., Gotze, S., Buchholz, K., Na'amnieh, S., Pawlowski, A. and Hecht, H. J. (2006). "Synthesis of sucrose analogues and the mechanism of action of *Bacillus subtilis* fructosyltransferase (levansucrase)." Carbohydr Res 341(14): 2335-49.

Songre-Ouattara, L. T., Mouquet-Rivier, C., Icard-Verniere, C., Humblot, C., Diawara, B. and Guyot, J. P. (2008). "Enzyme activities of lactic acid bacteria from a pearl millet fermented gruel (ben-saalga) of functional interest in nutrition." Int J Food Microbiol 128(2): 395-400.

Stolz, P., Böcker, G., Hammes, W. P. and Vogel, R. F. (1995). "Utilization of electron acceptors by lactobacilly isolated from sourdough." Z Lebensm Unters Forsch 201: 91-96.

Tieking, M., Ehrmann, M. A., Vogel, R. F. and Gänzle, M. G. (2005). "Molecular and functional characterization of a levansucrase from the sourdough

isolate *Lactobacillus sanfranciscensis* TMW 1.392." Appl Microbiol Biotechnol 66(6): 655-63.

Tieking, M., Korakli, M., Ehrmann, M. A., Gänzle, M. G. and Vogel, R. F. (2003). "In situ production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria." Appl Environ Microbiol 69(2): 945-52.

Trindade, M. I., Abratt, V. R. and Reid, S. J. (2003). "Induction of sucrose utilization genes from *Bifidobacterium lactis* by sucrose and raffinose." Appl Environ Microbiol 69(1): 24-32.

van Hijum, S. A., Kralj, S., Ozimek, L. K., Dijkhuizen, L. and van Geel-Schutten, I. G. (2006). "Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria." Microbiol Mol Biol Rev 70(1): 157-76.

Van Loo, J., Cummings, J., Delzenne, N., Englyst, H., Franck, A., Hopkins, M., Kok, N., Macfarlane, G., Newton, D., Quigley, M., Roberfroid, M., van Vliet, T. and van den Heuvel, E. (1999). "Functional food properties of non-digestible oligosaccharides: a consensus report from the ENDO project (DGXII AIRII-CT94-1095)." Br J Nutr 81(2): 121-32.

Yoon, M. Y. and Hwang, H. J. (2008). "Reduction of soybean oligosaccharides and properties of alpha-D-galactosidase from *Lactobacillus curvatus* R08 and *Leuconostoc mesenteroides* [corrected] JK55." Food Microbiol 25(6): 815-23.

2.6. Supplementary material

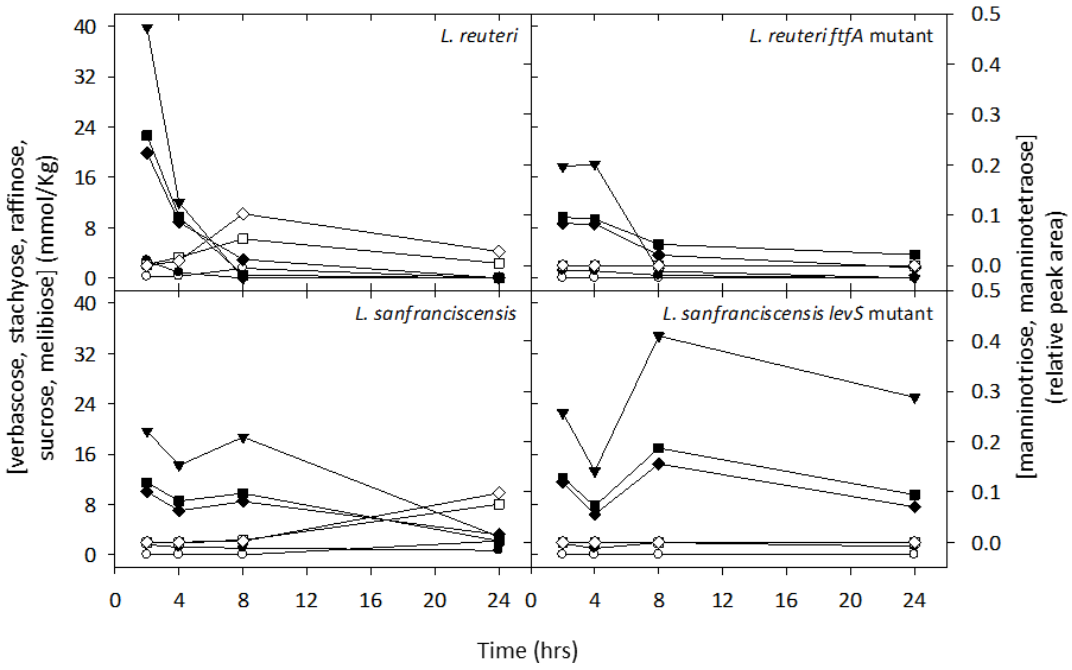


Figure 2-7. Carbohydrate concentrations in field pea fermentations with *L. reuteri* LTH5448, *L. sanfranciscensis* LTH2590 and their isogenic levansucrase-deficient mutants. Symbols indicate concentrations of sucrose (▼), melibiose (▲), raffinose (●), stachyose (■), verbascose (♦); relative peak area are shown for the α-GOS manninotriose (□), manninotetraose (◊). Data are qualitatively representative for two independent experiments with field pea flour.

CHAPTER THREE: **FUNCTIONAL CHARACTERIZATION OF SUCROSE PHOSPHORYLASE AND *SCRR*, A REGULATOR OF SUCROSE METABOLISM IN *LACTOBACILLUS REUTERI***

A version of this chapter has been published in Food Microbiology. Januana S. Teixeira, Reihaneh Abdi, Marcia Shu-Wei Su, Clarissa Schwab, and Michael G. Gänzle. (July 2013; in press)

CHAPTER 3

3.1. Introduction

Lactobacillus reuteri is a stable member of sourdough microbiota (Gänzle and Vogel, 2003; De Vuyst and Neysens, 2005) that occurs predominantly in industrial sourdough fermentations prepared for the production of baking improvers (Brandt, 2007) and in cereal fermentations in tropical climates (Vogel *et al.*, 1999). Sucrose is the most abundant fermentable carbon source in ungerminated cereal grains. Wheat and rye grains contain more than 0.6% and 1.2% sucrose, respectively, and sucrose is commonly used in bread formulas. Raffinose is additionally present in concentrations of 0.1 – 0.4% (Belitz *et al.*, 2004). The ability of *L. reuteri* to metabolize sucrose and raffinose as sole carbon sources contributes to its ecological fitness in food fermentations, and impacts the quality of fermented cereal products (Figure 3-1, Tieking *et al.*, 2005; Schwab *et al.*, 2007; Teixeira *et al.*, 2012).

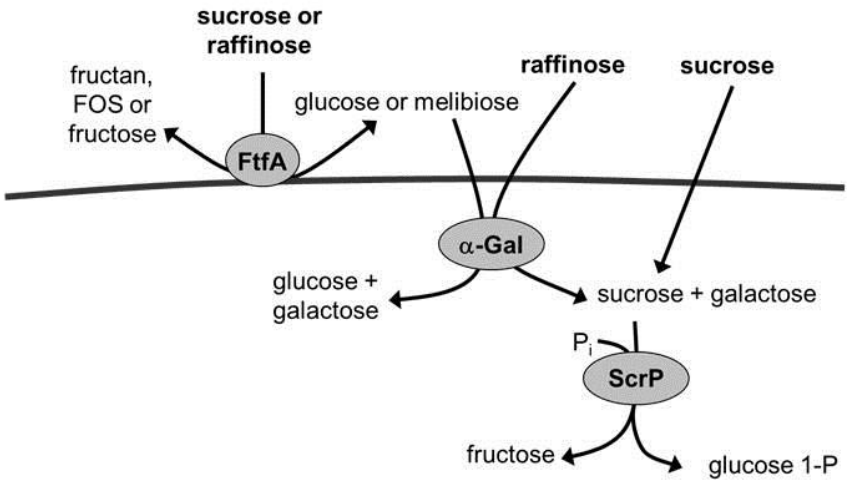


Figure 3-1. Sucrose and raffinose utilization in *L. reuteri* LTH5448 (modified from Teixeira *et al.*, 2012). FtfA, levansucrase, α-Gal, α-galactosidase, ScrP, sucrose phosphorylase. The scheme does not indicate the transport proteins involved in metabolism because transport proteins with specificity for sucrose, melibiose, or raffinose are not annotated in *L. reuteri* genomes, and have not been characterized at the biochemical level.

CHAPTER 3

Lactobacilli harbor a multitude of enzymes and transport systems involved in sucrose metabolism (Kaplan and Hutkins, 2003; Barrangou *et al.*, 2006; Goh *et al.*, 2006; van Hijum *et al.*, 2006; Saulnier *et al.*, 2007). Sucrose metabolism is mediated by β-fructofuranosidases (SacA or BfrA) catalyzing hydrolysis of sucrose or sucrose-phosphate (Saulnier *et al.*, 2007; Nakai *et al.*, 2012 for review,see Gänzle and Follador, 2012). The intracellular sucrose phosphorylase (ScrP) or extracellular glucansucrases and fructansucrases complement or substitute β-fructofuranosidases in few *Lactobacillus* spp. (van Hijum *et al.*, 2006; Gänzle and Follador, 2012). Fructansucrases but not glucansucrases also use raffinose, stachyose, and verbascose as substrates (van Hijum *et al.*, 2006; Teixeira *et al.*, 2012).

The expression of sucrose PTS systems is regulated by operon-specific (local) transcriptional regulators, in conjunction with the catabolite control protein A, CcpA, a transcriptional regulator of the LacI/GalR family (Andersson *et al.*, 2005; Reid and Abratt, 2005; Monedero *et al.*, 2007; Francke *et al.*, 2008). In *L. plantarum, L. paracasei* and *L. acidophilus*, sucrose metabolic genes are induced by sucrose or short chain fructo-oligosaccharides. MrmR / SacR were identified as putative local regulators of the sucrose catabolic operons on the basis of sequence similarities to other regulatory proteins (Goh *et al.*, 2006; Saulnier *et al.*, 2007). Taken together, these mechanisms mediate repression of carbon catabolite operons in the presence of more favorable carbon sources, and expression in the presence of the substrates.

Genome sequence data of more than 10 strains of *L. reuteri* in combination with functional analysis of sucrose metabolic enzymes demonstrate that sucrose utilization in this species is mediated by extracellular glucansucrases or fructansucrases and sucrose phosphorylase. Sucrose PTS systems or intracellular β-fructofuranosidases are absent (Schwab *et al.*, 2007; Frese *et al.*, 2011; Gänzle and Follador, 2012; Teixeira *et al.*, 2012). The expression of sucrose phosphorylase and levansucrase in *L. reuteri* is induced by sucrose but the expression of glucansucrases is not influenced by the carbon source (Schwab and Gänzle, 2006; Årsköld *et al.*, 2007). The regulation of sucrose catabolism in *L. reuteri* thus differs fundamentally from *L. plantarum* and *L. acidophilus*, the two species of lactobacilli for which experimental data is available (for review, see Gänzle and Follador, 2012). It was the aim of this study to characterize the regulation of sucrose metabolism in *L. reuteri*. The study employed *L. reuteri* LTH5448, a sourdough isolate harboring a sucrose phosphorylase (*scrP*) and a levansucrase (*ftfA*) (Schwab *et al.*, 2007; Teixeira *et al.*, 2012). The role of the putative sucrose regulator *scrR* was elucidated by quantification of the expression of sucrose metabolic genes in a *scrR* mutant and the wild type strain.

3.2. Materials and methods

3.2.1. Strains, media and growth conditions

Bacterial strains and plasmids used in this study are shown in Table 3-1. *Escherichia coli* JM109 (Promega, Nepean, Canada) was cultured in Luria-

---

Asterisks (*) indicate contributions by Dr. Clarissa Schwab.

Bertani (LB) broth at 37°C. *L. reuteri* strains were anaerobically cultivated at 37°C in modified deMan-Rogosa-Sharpe medium (mMRS) (Stolz *et al.*, 1995) or MRS containing 20 g L^{-1} sucrose (sucMRS); 20 g L^{-1} raffinose (rafMRS); 20 g L^{-1} fructo-oligosaccharides (fosMRS); and 20 g L^{-1} each of glucose and sucrose (glusucMRS) as carbon sources. Fructo-oligosaccharides were obtained from Orafti (Tienen, Belgium) with a degree of polymerization of 3 – 8. HPLC analysis verified that glucose, fructose and sucrose were essentially absent (data not shown). Other carbohydrates were purchased with 99% purity from Sigma (Oakville, Canada). The production of oligosaccharides and exopolysaccharides was also monitored in MRS containing 100 g L^{-1} sucrose (suc100MRS). Ampicillin (100 mg L^{-1}) or erythromycin (500 mg L^{-1}) was added to LB for selecting antibiotic-resistant *E. coli*. Erythromycin (10 mg L^{-1}) was added to mMRS medium to select erythromycin-resistant *L. reuteri*.

3.2.2. General molecular methods

Genomic DNA was isolated from overnight cultures grown in mMRS using DNeasy Blood & Tissue kit (Qiagen, Mississauga, Canada). DNA was amplified by polymerase chain reaction (PCR) using Taq DNA polymerase and dNTPs from Invitrogen (Burlington, Canada). Primer design for sequencing *scrR, scrP*, and the flanking regions of the genes was based on the genome sequence of *L. reuteri* 100-23. Primers were purchased from Integrated DNA Technologies (Coralville, USA) and are listed in Table 3-2. PCR products were visualized after electrophoretic separation on agarose gels. DNA sequencing was performed by Macrogen (MacrogenUSA, Rockville, USA).

Table 3-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
	Strains	
E. coli JM109	Cloning host for pGEMTeasy- and pJRS233-derivative plasmids	Promega
L. reuteri LTH5448	Sourdough isolate	Schwab *et al.* 2007
L. reuteri LTH5448Δ*scrP*	Wild type strain derivative with a deletion in *scrP*	This study
L. reuteri LTH5448Δ*scrR*	Wild type strain derivative with a deletion in *scrR*	This study
	Plasmids	
pGEMTeasy	Cloning vector used in *E. coli*; 3.0 kb; Amp^r	Promega
pScrP-A	pGEMTeasy containing a 0.9 kb sequence upstream of *scrP*; 3.9 kb; Amp^r	This study
pScrP-B	pGEMTeasy containing a 0.9 kb sequence downstream of *scrP*; 3.9 kb; Amp^r	This study
pScrP-AB	pGEMTeasy containing the upstream and downstream sequences of *scrP*; 4.8 kb; Amp^r	This study
pScrR-A	pGEMTeasy containing a 1.2 kb sequence upstream of *scrR*; 4.2 kb; Amp^r	This study
pScrR-B	pGEMTeasy containing a 1.2 kb sequence downstream of *scrR*; 4.2 kb; Amp^r	This study
pScrR-AB	pGEMTeasy containing the upstream and downstream sequences of *scrR*; 5.4 kb; Amp^r	This study
pJRS233	Shuttle vector used in the hosts *E. coli* and *L. reuteri*; 6.0 kb; Erm^r	Perez-Casal *et al.* 1993
pKO-*scrP*-AB	pJRS233 containing 1.8 kb of the flanking sequences of *scrP*; 7.8 kb; Erm^r	This study
pKO-*scrR*-AB	pJRS233 containing 2.4 kb of the flanking sequences of *scrR*; 8.4 kb; Erm^r	This study

Amr^r, Erm^r, ampicillin and erythromycin resistance, respectively

**3.2.3. Phylogenetic analysis of scrR*

Sucrose regulators and CcpA were aligned using CLUSTALW implemented in BioEdit. Phylogenetic analysis of ScrR sequences was performed using MEGA (Tamura *et al.*, 2011) and the maximum likelihood method applying the Jones-Taylor-Thornton (JTT) substitution model. Bootstrap support was calculated for 100 replicates.

3.2.4. Generation and verification of L. reuteri *LTH5448Δ*scrP *and LTH5448Δ*scrR *mutants*

Sequencing of *scrP* and *scrR* genes in *L. reuteri* LTH548 was achieved with primers specific for the corresponding loci in the genome sequenced strain *L. reuteri* 100-23 (Gene bank Accession number AAPZ00000000.2, primers listed in Table 3-2). In-frame truncation of *scrP* and *scrR* genes was achieved with the temperature-sensitive shuttle vector pJRS233 (Su *et al.*, 2011). In brief, flanking fragments of the target genes were amplified from genomic DNA of *L. reuteri* LTH5448 by PCR (primers are listed in Table 3-2). The amplicons were ligated into pGEMTeasy vector (Promega) to generate pScrP-A, pScrP-B, pScrR-A, and pScrR-B (Table 3-1). The flanking fragments of *scrP* and *scrR* from these plasmids were digested with the respective restriction enzymes (New England Biolabs, Pickering, Canada), purified, and ligated into pGEMTeasy to create pScrP-AB and pScrR-AB. The co-ligated DNA fragments in pScrP-AB and pScrR-AB were cut with the respective restriction enzymes, and ligated into pJRS233 using T4 DNA ligase (Epicentre, Markham, Canada). The resulting plasmids pKO-*scrP*-AB and pKO-*scrR*-AB were electrotransformed into competent *L. reuteri* LTH5448 cells suspended in water with 30% (v/v) polyethylene glycol (MW 3350; J.T. Baker Chemical, Phillipsburg, NJ). Transformants were grown in mMRS broth containing erythromycin at 42°C for 80 generations to select for single crossover mutants. *L. reuteri* with pKO-*scrP*-AB and pKO-*scrR*-AB integrated into chromosome were cured by culturing in mMRS broth at 37°C for 100 generations. Erythromycin-sensitive double

crossover mutants were identified by replica plating on mMRS and mMRS-erythromycin agar. The in-frame deletion of *scrP* and *scrR* in *L. reuteri* LTH5448Δ*scrP* and *L. reuteri* LTH5448Δ*scrR*, respectively, was confirmed by PCR. DNA sequencing was conducted to verify the deletion region using sequencing primers (Table 3-2).

Table 3-2. Oligonucleotide primers used in this study

Gene	Primer	Sequence (5' – 3')[a]	Application
scrP	*pho*-KO1b-F-*Xho*I	CCG*CTGGA*GGTATGAAGGTACAACC	Insert primer
	pho-KO2-R-*Xba*I	GC*TCTAGA*TTAGTCAGAGTAAGTAATTAAC	Insert primer
	pho-KO3-F-*Xba*I	GC*TCTAGA*TTCACTATTACAGCTAACGGCGAA	Insert primer
	pho-KO4a-R-*Pst*I	AA*CTGCAG*GCCTGGGAATTTCTACCC	Insert primer
	pho-1b-F	GTATGAAGGTACAACC	Sequencing
	pho-4a-R	GCTTGGGAATTTCTACCC	Sequencing
	SucPho F	CTGGTAAGAACCGTCCAAC[b]	qPCR
	SucPho R	CAGTTAAGATATCCTTAGCAT[b]	qPCR
scrR	*reg*-KO1a-F-*Xho*I	CCG*CTCGAG*TAATTCCAAATAGGTTATG	Insert primer
	reg-KO2-R-*BamH*I	CG*GGATCC*TCATTGAGCAACATCTTTTAAC	Insert primer
	reg-KO3-F-*BamH*I	CG*GGATCC*TAAACTTGTTCCGGTTGA	Insert primer
	reg-KO4b-R-*Hind*III	CCC*AAGCTT*CTTCCAATGGTCAAAT	Insert primer
	reg-5-F	CCGTCGATTGTTCAAATATG	Sequencing
	reg-6-R	CTGTTGAACGAATTCACG	Sequencing
	reg-9-F	TGGGGATAGAGGAATATCATC	qPCR
	reg-10-R	CGATTGATGGTAGGTGAAAC	qPCR
pho	Phoket V	GTAACCTTCAAGGAATCC[b]	qPCR
	Phoket R	CGTCTTTACGCATTCCTTG[b]	qPCR
ftfA	Leureu1001 V	GAATGGCTATCAACTTGTG[b]	qPCR
	Leureu1001 R	CTTCTACTTGCGGGTTC[b]	qPCR

[a] Restriction enzyme sites are indicated in italic form

[b] Primers from Schwab et al., 2007; other primers were designed in this study

3.2.5. Isolation of mRNA and synthesis of cDNA libraries

RNA was isolated from exponentially growing cells (OD_{600} of 0.5) of *L. reuteri* LTH5448 and its mutant LTH5448Δ*scrR* in mMRS, gluMRS, sucMRS, rafMRS or glusucMRS. Two volumes of RNAprotect (Qiagen) were added, and cells were harvested by centrifugation at 5000 × *g*. Cells were resuspended in 1 mL TRIzol (Invitrogen), and disrupted using a bead beater (Biospec products, Bartlesville, USA) and 0.1 mm zirconia-silica beads. The supernatant was transferred to 1.5 mL Eppendorf tubes for RNA isolation according to TRIzol manufacturer's manual. Reagents and enzymes for DNase I treatment and reverse transcription were purchased from Promega (Madison, USA) and used according to the manufacturer's instructions. Transcription of *scrP*, *scrR,* and *ftfA* in wild type *L. reuteri* LTH5448 grown in mMRS and sucMRS was analysed by PCR using the primers shown in Table 3-2.

3.2.6. Quantification of transcriptional levels of the scrP, scrR *and* ftfA *genes in response to glucose, sucrose, and raffinose*

Quantitative analysis of the expression of *scrP*, *scrR,* and *ftfA* in *L. reuteri* LRH5448 and LTH5448Δ*scrR* was carried out using cDNA as a template in quantitative PCR (qPCR) reactions. DNase-I treated mRNA preparations were used as negative control; chromosomal DNA was used as positive control. Amplifications were carried out in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with primers listed in Table 3-2. Melting curve analysis and determination of amplicon size by agarose gel electrophoresis

verified the specific amplification of the appropriate cDNA. Expression levels of *scrP*, *scrR* and *ftfA* in cultures grown in mMRS, gluMRS, rafMRS or glusucMRS were calculated by the $\Delta\Delta C_T$ method according to Pfaffl (2001). The phosphoketolase gene (*pho)* was used as housekeeping gene. Maltose and / or sucrose rather than glucose are preferred carbon sources for *L. reuteri* (Stolz *et al.*, 1993; Schwab *et al.*, 2007); therefore, cultures grown in sucMRS were used as reference conditions. Gene expression was quantified in duplicate from cDNA libraries obtained from three (*scrP* and *ftfA*) or two (*scrR*) independent experiments.

3.2.7. Determination of growth kinetics and metabolite formation

Growth of *L. reuteri* LTH5448, LTH5448Δ*scrP*, and LTH5448Δ*scrR* was assessed in mMRS, sucMRS, rafMRS or fosMRS. Media were inoculated with 1% of washed overnight cultures, incubated at 37°C, and growth was monitored by changes in the optical density (OD) at 600 nm over 24 hours. Samples were taken for metabolite analysis by HPLC as described (Teixeira *et al.*, 2012). Data shown are mean values for duplicate (*L. reuteri* LTH5448 and LTH5448Δ*scrP*) or triplicate (LTH5448Δ*scrR*) independent experiments.

Oligosaccharide and levan formation by *L. reuteri* LTH5448 and LTH5448Δ*scrP* was assessed in suc100MRS. Media were inoculated with overnight cultures and incubated at 37°C. Samples were taken for analysis by HPAEC-PAD and size exclusion chromatography as described (Galle *et al.*, 2010). The amount of high molecular weight levan (relative molecular weight of

more than 10^5) in suc100MRS was calculated using levan from *L. reuteri* LTH5448 purified by ethanol precipitation, dialysis, and phenol-chlorofom extraction (Wang *et al.*, 2010) as standards. Levan with intermediate molecular weight (10^4 – 10^5) was not quantified in culture supernatants due to interference of media components. Data shown are mean values for duplicate (FOS) or triplicate (levan) independent experiments.

Polysaccharides from culture and supernatant were hydrolyzed with 2M H_2SO_4 at 80°C for 2 hours in order to differentiate bound levan from levan released into the culture medium. The monosaccharides obtained from acid hydrolysis were quantified with an Aminex 87H column as described (Schwab *et al.*, 2007). To assess levan hydrolysis by *L. reuteri* LTH5448 and LTH5448Δ*ftfA*, cells from overnight cultures were washed with 50 mmol L^{-1} phosphate buffer (pH 6.5) and incubated with 5 g L^{-1} levan for 24 h. Samples were taken periodically and levan was quantified by size exclusion chromatography as described above. The incubation in phosphate buffer avoids interference with media components, and thus allows quantification of levan with intermediate molecular weight (molecular weight range 10^4 -> 10^6).

3.2.8. Nucleotide Accession numbers

The nucleotide sequences of *scrP* and *scrR* in *L. reuteri* LTH5448, and the sequences of truncated *scrP* and *scrR* in *L. reuteri* LTH5448Δ*scrP* and *L. reuteri* LTH5448Δ*scrR* are deposited in GenBank (Accession number KC539460, KC539461, KC539462, and KC539463, respectively).

3.3. Results

*3.3.1. Identification of genes coding for sucrose phosphorylase and a putative sucrose dependent regulator in L. reuteri LTH5448

A partial sequence of *scrP* coding for sucrose phosphorylase was previously identified in *L. reuteri* LTH5448 (Schwab *et al.*, 2007). Sequencing of this gene and its flanking regions confirmed the presence of a sucrose phosphorylase in *L. reuteri* LTH5448. The gene sequence and the genetic organization (Figure 3-2A) are homologous to *L. reuteri* 100-23.

Bioinformatic analysis of the genome of *L. reuteri* 100-23 identified the putative sucrose regulator *scrR*. The *scrR* gene was flanked by divergently oriented muramidase and a hypothetical protein (Figure 3-2B) and located distantly from *scrP* or *ftfA*. An *scrR* gene (length 942 base pairs) was amplified by

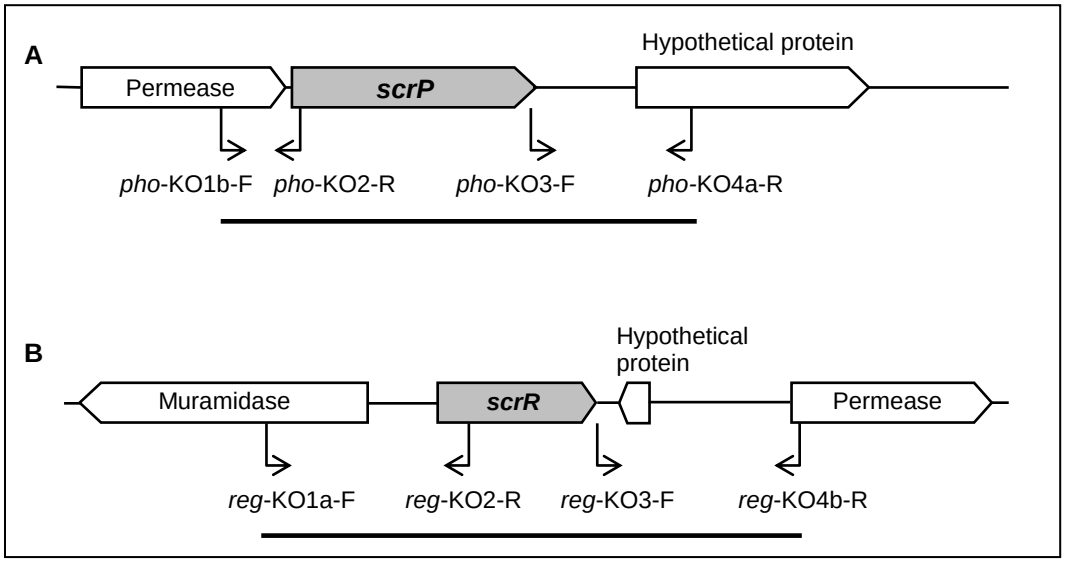


Figure 3-2. Gene loci encoding sucrose phosphorylase (*scrP*) (Panel A) and its putative regulator (*scrR*) (Panel B) in *L. reuteri* 100-23 (Gene bank Accession number AAPZ00000000.2). The lines below represent the homologous sequences in *L. reuteri* LTH5448. PCR primers used for the generation of LTH5448Δ*scrR* and LTH5448Δ*scrP* mutants are indicated by arrows.

PCR in *L. reuteri* LTH5448. In *L. reuteri* LTH5448, the coding region of *scrR* and *scrP* and their flanking sequences were highly homologous to *L. reuteri* 100-23. The prediction of putative regulator ScrR encodes 314 amino acid residues. The gene is present in all sequenced genomes of *L. reuteri* strains with 96-98% homology, and is highly homologous to sucrose regulators of *Streptococcus mutans* (ScrR, U 46902) (48% identity in 307 aa) and *L. acidophilus* NCFM (MsmR, 68% identity in 141 aa). ScrR of *L. reuteri* clustered with sucrose regulators of other lactic acid bacteria and bifidobacteria in a cluster distant from the global regulator CcpA (Figure 3-3). The putative sucrose regulator ScrR possesses the N-terminal helix-

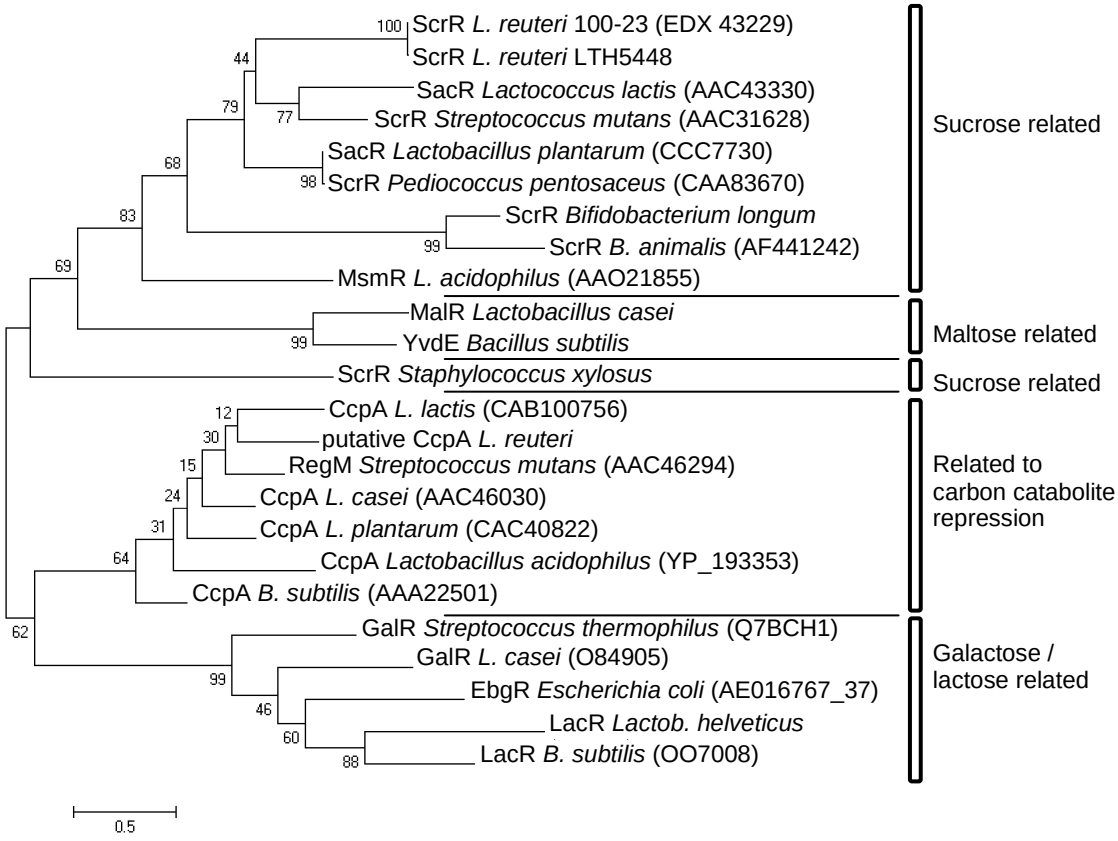


***Figure 3-3.** Phylogenetic analysis of sucrose specific regulator ScrR and related transcriptional factors in the LacI/GalR family. The distance tree was constructed using the maximum likelihood method. Bootstrap values were calculated with 100 replicates.

-turn helix (HTH) DNA binding domain of the LacI-GalR-type transcriptional regulators, and the core unit with a motive indicative of periplasmic binding proteins and the sugar binding domain of the LacI family. Qualitative analysis of cDNA libraries of *L. reuteri* LTH54448 indicated that *scrR* is expressed in *L. reuteri* LTH5448 (data not shown).

3.3.2. Generation of scrR *and* scrP *deletion mutants of* L. reuteri *LTH5448*

To identify the regulatory role of *scrR* in transcription of *scrP* and *ftfA,* and the impact of *scrP* on sucrose utilization, double crossover mutants of *L. reuteri* LTH5448 were generated. The truncation in *scrP* of *L. reuteri* LTH5448Δ*scrP* was confirmed by PCR with the primers *pho*-KO1b-F-*Xho*I and *pho*-KO4b-R-*Pst*I (Table 3-2). PCR amplified the expected amplicons of 3.2 kb and 1.8 kb with DNA from *L. reuteri* LTH5448 and LTH5448Δ*scrP*, respectively, as template. A deletion in *scrR* of *L. reuteri* LTH5448Δ*scrR* was verified by PCR with the primers *reg*-KO1a-F-*XhoI* and *reg*-KO4b-R-*HindIII* (Table 3-2), yielding amplicons of 3.3 kb and 2.6 kb for *L. reuteri* LTH5448 and LTH5448Δ*scrR,* respectively. DNA sequencing verified the truncation of *scrP* and *scrR* in *L. reuteri* LTH5448Δ*scrP* and LTH5448Δ*scrR,* respectively.

3.3.3. Expression of scrP *and* scrR *in* L. reuteri *LTH5448 and LTH5448*ΔscrR

The transcription of *scrP, scrR,* and *ftfA* in wild type *L. reuteri* and its LTH5448Δ*scrR* mutant in response to glucose, sucrose, and raffinose was quantified relatively to its expression in sucMRS (Table 3-3 and data not shown).

The wild type strain showed comparable expression of *scrR* when grown with or without sucrose (data not shown). *ScrP* was expressed in presence of sucrose and raffinose, but was not repressed by glucose when sucrose was present. In contrast to the wild type strain, the LTH5448Δ*scrR* mutant strain showed expression of *scrP* when grown without sucrose (Table 3-3).

Table 3-3. Sucrose phosphorylase and levansucrase expression in *L. reuteri* LTH5448 and LTH5448Δ*scrR*. Data are shown as means ± standard deviation of triplicate independent experiments.

***L. reuteri* strain**	**Target gene**	**Sugar composition of media**			
		Glucose, Maltose, Fructose	**Glucose**	**Raffinose**	**Glucose, Sucrose**
		Log_{10} [relative gene expression]			
LTH5448	*scrP*	-2.17 ± 0.45	-2.76 ± 0.29	0.18 ± 0.47	0.11 ± 0.36
LTH5448Δ*scrR*	*scrP*	0.02 ± 0.23	0.58 ± 0.21	0.41 ± 0.23	0.36 ± 0.86
LTH5448	*ftfA*	-1.87 ± 0.27	-1.61 ± 1.12	0.64 ± 0.45	0.31 ± 0.58
LTH5448Δ*scrR*	*ftfA*	-0.67 ± 0.53	-0.20 ± 0.77	-0.17 ± 0.29	-0.29 ± 0.50

The levanuscrase FtfA was also highly expressed in the presence of sucrose but expression levels were much lower when sucrose was absent. Comparable to the expression of *scrP*, expression of *ftfA* in *L. reuteri* LTH5448 was not repressed by glucose and deletion of *scrR* resulted in constitutive expression of the enzyme (Table 3-3). These data demonstrate that *scrR* codes for a transcriptional regulator of the LacI/GalR-family that mediates repression of sucrose metabolic genes in the absence of sucrose.

3.3.4. Growth kinetics and carbohydrate metabolism in mMRS

In order to determine the contribution of *scrP* and *scrR* to sucrose metabolism, growth and carbohydrate metabolism of *L. reuteri* LTH5448 and its mutants were compared in media with different carbon sources. There were no differences in the growth of *L. reuteri* LTH5448, LTH5448Δ*scrP* and LTH5448Δ*scrR* during growth in mMRS, sucMRS or rafMRS (Figure 3-4).

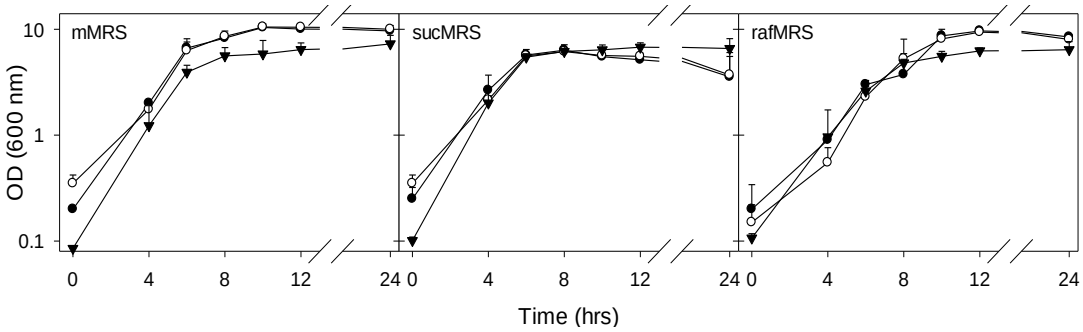


Figure 3-4. Growth of *L. reuteri* LTH5448 (●), LTH5448Δ*scrP* (○) and LTH5448Δ*scrR* (▼) with maltose, glucose and fructose (mMRS); sucrose (sucMRS); or raffinose (rafMRS) as substrate. Data shown are means ± standard deviation of duplicate (*L. reuteri* LTH5448 and LTH5448Δ*scrP*) and triplicate (LTH5448Δ*scrR*) independent experiments.

Growth and metabolism of *L. reuteri* LTH5448 and LTH5448Δ*scrR* were also compared in gluMRS. The results confirmed that disruption of *scrR* does not result in a phenotype that is detectable by metabolite analysis (data not shown). Samples obtained at 24 hours of fermentation showed that all strains completely metabolized the sugars in all three media (data not shown), formed comparable amounts of lactate, acetate, and ethanol, and reduced comparable amounts of fructose to mannitol (Table 3-4). These similarities can be explained by the fact that the expression of sucrose metabolic genes is not different between *L. reuteri*

LTH5448 and LTH5448Δ*scrR* when the substrate is present. In the absence of the substrate, the expression of sucrose metabolic genes in *L. reuteri* LTH5448Δ*scrR* does not influence sucrose metabolism. In *L. reuteri* LTH5448Δ*scrP*, FtfA activity compensates for the absence of ScrP. In fosMRS, all strains grew poorly and did not produce considerable amounts of organic acids (data not shown).

Table 3-4. Formation of organic acids of *L. reuteri* LTH5448 and LTH5448Δ*scrP* mutant during growth in mMRS with maltose, glucose and fructose; sucrose (sucMRS); or raffinose (rafMRS) as substrate. Data are shown as means ± standard deviation of duplicate independent experiments.

Medium	***L. reuteri* strain**	**Metabolites [mmol L^{-1}]**			
		Mannitol	**Lactate**	**Acetate**	**Ethanol**
mMRS	LTH5448	18 ± 0	77 ± 4	10 ± 1	58 ± 6
	Δ*scrP*	19 ± 1	76 ± 4	10 ± 1	53 ± 7
sucMRS	LTH5448	14 ± 0	26 ± 0	11 ± 0	10 ± 2
	Δ*scrP*	14 ± 1	25 ± 2	10 ± 2	10 ± 1
rafMRS	LTH5448	15 ± 0	61 ± 3	10 ± 2	49 ± 1
	Δ*scrP*	18 ± 2	62 ± 1	10 ± 1	46 ± 4

3.3.5. Synthesis of levan and fructo-oligosaccharides

L. reuteri LTH5448 and LTH5448Δ*scrP* produced FOS and levan during growth in suc100MRS. Wild type and LTH5448Δ*scrP* produced four different FOSs (FOS_1-FOS_4) (Figure 3-5). Maximum levan levels were 4.6 g L^{-1} in *L. reuteri* LTH5448 and 6.7 g L^{-1} in LTH5448Δ*scrP* after 6 and 8 hours of fermentation, respectively (Figure 3-5B). Levan concentration increased during the exponential phase of growth followed by an apparent decrease during subsequent incubation. The quantification of total fructans after hydrolysis of levan and FOS with H_2SO_4

indicated that this decrease is attributable to partial hydrolysis of levan to polysaccharides with intermediate molecular weight that were not accounted by the analytical tools employed. Indeed, incubation of *L. reuteri* LTH5448 or LTH5448Δ*ftfA* with purified levan in phosphate buffer resulted in a reduced molecular weight of levan but not in hydrolysis to fructose or FOS, excluding that FtfA was responsible for the decrease in levan concentration during stationary growth phase (data not shown).

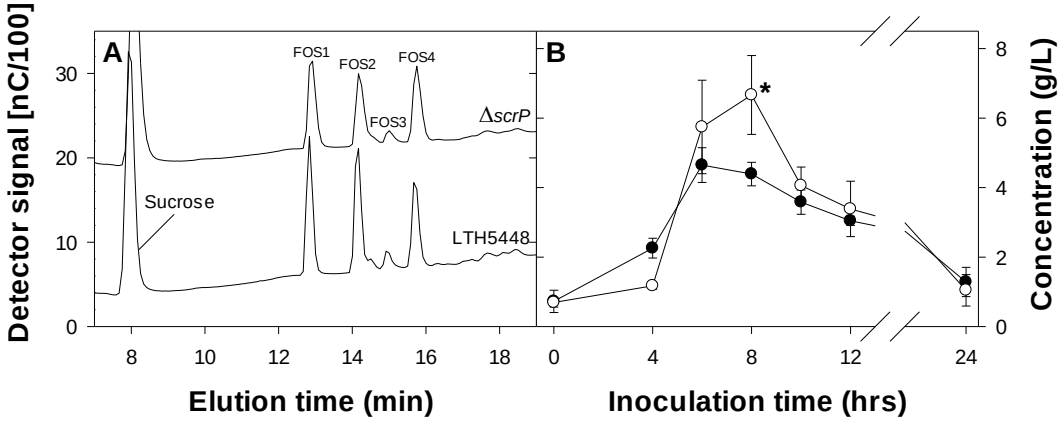


Figure 3-5. Formation of fructooligosaccharides and levan during growth of *L. reuteri* LTH5448 and LTH5448Δ*scrP* in presence of 100 g L^{-1} sucrose. Panel A. FOS formation was quantified after 24 hours of incubation. Panel B. Levan concentration in the culture supernatant of *L. reuteri* LTH5448 (●) and LTH5448Δ*scrP* (○). Data are shown as means ± standard deviation of three independent experiments. Significant differences ($p < 0.001$) between the levan concentrations in *L. reuteri* LTH5448 and LTH5448Δ*scrP* were determined by Student's *t*-test and are indicated by an asterisk.

3.4. Discussion

A multitude of enzymes, transport systems, and regulatory elements mediate sucrose metabolism in lactobacilli (Goh *et al.*, 2006; van Hijum *et al.*, 2006; Saulnier *et al.*, 2007). Metabolic pathways for the metabolism of sucrose and fructo-oligosaccharides partially overlap (Kaplan and Hutkins, 2003; Goh *et*

al., 2006; Saulnier *et al.*, 2007; Gänzle and Follador, 2012). Transcriptome analysis in *L. acidophilus* suggested that metabolism of sucrose but not metabolism of FOS in *L. acidophilus* is likely mediated by a sucrose PTS system (Barrangou *et al.*, 2006). Functional analysis of sucrose metabolism in *L. plantarum* and *L. paracasei* demonstrated that transport and hydrolysis of sucrose as well as short chain FOS are mediated by phosphotransferase systems and associated (phospho)-β-fructofuranosidases (Goh *et al.*, 2006; Saulnier *et al.*, 2007).

In *L. reuteri* LTH5448, sucrose metabolism occurs via two alternative enzymes, the extracellular levansucrase FtfA and the intracellular sucrose phosphorylase ScrP. Sucrose phosphorylase is inducible by sucrose and raffinose in *L. reuteri*, *L. acidophilus* and *Bifidobacterium animalis* (Trindade *et al.*, 2003; Barrangou *et al.*, 2006; Schwab *et al.*, 2007; Teixeira *et al.*, 2012) but does not recognize FOS or raffinose as substrate (Kawasaki *et al.*, 1996; van den Broek *et al.*, 2004). The induction of ScrP by raffinose is thus likely mediated by the intracellular release of sucrose from raffinose by intracellular α-galactosidase activity (Yoon and Hwang, 2008; Teixeira *et al.*, 2012).

Sucrose and raffinose utilization by *L. reuteri* LTH5448 was not impaired by deletion of sucrose phosphorylase as ScrP and FtfA are equally suited to support rapid turnover of sucrose (Schwab *et al.*, 2007, this study). Remarkably, *L. reuteri* LTH5448Δ*scrP* produced about 25% more levan, confirming that the lack of sucrose phosphorylase is compensated by increased turnover by levansucrase as sole sucrose converting enzyme. Formation of 3.3 g levan / kg by

L. reuteri during growth in sourdough improved bread volume, and delayed staling (Galle *et al.,* 2012); the use of sucrose phosphorylase-negative strains may thus be beneficial in applications aiming at a high yield of polymeric fructan.

This study identified a putative sucrose regulator ScrR of *L. reuteri* with homology to regulatory proteins of the GalR-LacI family including MsmR and SacR. Structurally related ScrR regulate the (phospho)-β-fructofuranosidases ScrA and enzymeII^{suc} of sucrose PTS systems in *S. mutans, P. pentosaceus, L. lactis* (Figure 3-3) and sucrose phosphorylases in *Bifidobacterium lactis* (Gering and Bruckner, 1996; Hiratsuka *et al.,* 1998; Luesink *et al.,* 1999; Trindade *et al.,* 2003). In contrast to *L. plantarum* and *L. acidophilus,* genes coding for sucrose metabolic enzymes and their regulation in *L. reuteri* are not encoded on a single operon but are on three distinct genetic loci. Moreover, sucrose metabolic enzymes in *L. reuteri* do not support catabolism of fructo-oligosaccharides other than sucrose but contribute to metabolism of raffinose-family oligosaccharides (Teixeira *et al.,* 2012; this study).

Inactivation of *scrR* in *L. reuteri* LTH5448 resulted in constitutive transcription of *ftfA* and *scrP* when the strain was grown in the presence of media containing glucose, indicating that *scrR* is a transcriptional repressor similar to *scrR* of *L. lactis, S. mutans,* and *Staphylococcus xylosus* (Gering and Bruckner, 1996; Hiratsuka *et al.,* 1998; Luesink *et al.,* 1999). Regulation of gene expression by *scrR* in *L. reuteri* is thus substrate controlled, comparable to the function of *scrR* of *B. animals* (Trindade *et al.,* 2003) and *S. mutans, msmR* and *scrR* of *L. acidophilus* and *L. plantarum,* respectively. Moreover, *scrR* in *L. reuteri*

regulated the expression of *ftfA*. Regulation of fructansucrases by *scrR* was not observed in past studies on transcriptional regulators of sucrose metabolism in lactic acid bacteria. In *L. sanfranciscensis*, levansucrase is the only enzyme capable of sucrose hydrolysis and is constitutively expressed (Tieking *et al.*, 2005). In addition to its contribution to sucrose and raffinose metabolism in *L. reuteri*, levansucrase also provides protection against environmental stressors and its expression is induced by heat stress and sublethal concentrations of proton-ionophores (Schwab and Gänzle, 2006). The finding that *ftfA* is regulated by *scrR* adds to our understanding of the multiple roles of levansucrases in the ecology of *L. reuteri* (Gänzle and Schwab, 2009).

In conclusion, *L. reuteri* takes a very different approach to utilize sucrose and to regulate sucrose metabolic genes when compared to its closely related organisms. Lactobacilli have adapted to nutrient rich habitats by reduction of the genome size (Makarova and Koonin, 2007). Among lactobacilli, *L. plantarum* has retained a relatively large genome size and the ability to metabolise a large spectrum of carbohydrates, and is found in diverse habitats (Makarova and Koonin, 2007; Siezen and van Hylckama Vlieg, 2011). Glucose is metabolized preferentially over maltose or sucrose, reflecting carbon catabolite repression (Andersson *et al.*, 2005; Saulnier *et al.*, 2007). *L. reuteri* underwent a more extensive reductive evolution to adapt to specific vertebrate hosts (Walter, 2008; Frese *et al.*, 2011). In these narrow ecological niches, particularly the upper intestine of (grain-feeding) animals, sucrose and raffinose are major carbohydrate sources. This study demonstrates that sucrose metabolism in *L. reuteri* is not

subject to repression by glucose. *L. reuteri* apparently has abandoned regulatory circuits to repress sucrose metabolic enzymes in presence of glucose. This preferential metabolism of sucrose contributes to the ecological fitness of *L. reuteri* (Walter *et al.*, 2008; Sims *et al.*, 2011). Differences in the regulation of sucrose metabolism between *L. reuteri* and other lactobacilli thus reflect the ecological adaptation of *L. reuteri.*

Sourdough isolates of *L. reuteri* do not represent a separate, extra-intestinal evolutionary lineage of *L. reuteri* but are of intestinal origin (Su *et al.*, 2012). The concurrent expression of sucrose (and maltose) metabolic enzymes matches the carbohydrate availability in cereal fermentations, and accounts for the simultaneous utilization of carbohydrates during growth in sourdough (Gänzle *et al.*, 2007, this study). The simultaneous utilization of maltose and sucrose also determines the formation of the exopolysaccharides reuteran and levan as well as acetate, and thus has a substantial impact on bread quality (Tieking *et al.*, 2005; Kaditzky *et al.*, 2008; Galle *et al.*, 2012).

3.5. References

Andersson, U., Molenaar, D., Radstrom, P. and de Vos, W. M. (2005). "Unity in organisation and regulation of catabolic operons in *Lactobacillus plantarum*, *Lactococcus lactis* and *Listeria monocytogenes*." Syst Appl Microbiol 28(3): 187-95.

Årsköld, E., Svensson, M., Grage, H., Roos, S., Radstrom, P. and van Niel, E. W. (2007). "Environmental influences on exopolysaccharide formation in *Lactobacillus reuteri* ATCC 55730." Int J Food Microbiol 116(1): 159-67.

Barrangou, R., Azcarate-Peril, M. A., Duong, T., Conners, S. B., Kelly, R. M. and Klaenhammer, T. R. (2006). "Global analysis of carbohydrate utilization by *Lactobacillus acidophilus* using cDNA microarrays." Proc Natl Acad Sci U S A 103(10): 3816-21.

Belitz, H. D., Grosch, W. and Schieberle, P. (2004). Food Chem. Berlin, Springer.

Brandt, M. J. (2007). "Sourdough products for convenient use in baking." Food Microbiol 24(2): 161-4.

De Vuyst, L. and Neysens, P. (2005). "The sourdough microflora: biodiversity and metabolic interactions." Trends Food Sci Technol 16(1,Äì3): 43-56.

Francke, C., Kerkhoven, R., Wels, M. and Siezen, R. J. (2008). "A generic approach to identify Transcription Factor-specific operator motifs; Inferences for LacI-family mediated regulation in *Lactobacillus plantarum* WCFS1." BMC Genomics 9: 145.

Frese, S. A., Benson, A. K., Tannock, G. W., Loach, D. M., Kim, J., Zhang, M., Oh, P. L., Heng, N. C., Patil, P. B., Juge, N., Mackenzie, D. A., Pearson, B. M., Lapidus, A., Dalin, E., Tice, H., Goltsman, E., Land, M., Hauser, L., Ivanova, N., Kyrpides, N. C. and Walter, J. (2011). "The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri*." PLoS Genet 7(2): e1001314.

Galle, S., Schwab, C., Arendt, E. and Gänzle, M. (2010). "Exopolysaccharide-forming *Weissella* strains as starter cultures for sorghum and wheat sourdoughs." J Agric Food Chem 58(9): 5834-41.

Galle, S., Schwab, C., Dal Bello, F., Coffey, A., Gänzle, M. G. and Arendt, E. K. (2012). "Influence of in-situ synthesized exopolysaccharides on the quality of gluten-free sorghum sourdough bread." Int J Food Microbiol 155(3): 105-12.

Gänzle, M. and Schwab, C. (2009). "Ecology of exopolysaccharide formation by lactic acid bacteria: sucrose utilization, stress tolerance, and biofilm formation." Bacterial polysaccharides. M. Ullrich. Norfolk, Caister Academic Press: 263-278.

Gänzle, M. G. and Follador, R. (2012). "Metabolism of oligosaccharides and starch in lactobacilli: a review." Front Microbiol 3: 340.

Gänzle, M. G., Vermeulen, N. and Vogel, R. F. (2007). "Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough." Food Microbiol 24(2): 128-38.

Gänzle, M. G. and Vogel, R. F. (2003). "Contribution of reutericyclin production to the stable persistence of *Lactobacillus reuteri* in an industrial sourdough fermentation." Int J Food Microbiol 80(1): 31-45.

Gering, M. and Bruckner, R. (1996). "Transcriptional regulation of the sucrase gene of *Staphylococcus xylosus* by the repressor ScrR." J Bacteriol 178(2): 462-9.

Goh, Y. J., Zhang, C., Benson, A. K., Schlegel, V., Lee, J. H. and Hutkins, R. W. (2006). "Identification of a putative operon involved in fructooligosaccharide utilization by *Lactobacillus paracasei.*" Appl Environ Microbiol 72(12): 7518-30.

Hiratsuka, K., Wang, B., Sato, Y. and Kuramitsu, H. (1998). "Regulation of sucrose-6-phosphate hydrolase activity in *Streptococcus mutans*: characterization of the scrR gene." Infect Immun 66(8): 3736-43.

Kaditzky, S., Seitter, M., Hertel, C. and Vogel, R. (2008). "Performance of *Lactobacillus sanfranciscensis* TMW 1.392 and its levansucrase deletion mutant in wheat dough and comparison of their impact on bread quality." Eur Food Res Technol 227(2): 433-442.

Kaplan, H. and Hutkins, R. W. (2003). "Metabolism of fructooligosaccharides by *Lactobacillus paracasei* 1195." Appl Environ Microbiol 69(4): 2217-22.

Kawasaki, H., Nakamura, N., Ohmori, M. and Sakai, T. (1996). "Cloning and expression in *Escherichia coli* of sucrose phosphorylase gene from *Leuconostoc mesenteroides* No. 165." Biosci Biotechnol Biochem 60(2): 322-4.

Luesink, E. J., Marugg, J. D., Kuipers, O. P. and de Vos, W. M. (1999). "Characterization of the divergent *sacBK* and *sacAR* operons, involved in sucrose utilization by *Lactococcus lactis*." J Bacteriol 181(6): 1924-6.

Makarova, K. S. and Koonin, E. V. (2007). "Evolutionary genomics of lactic acid bacteria." J Bacteriol 189(4): 1199-208.

Monedero, V., Maze, A., Boel, G., Zuniga, M., Beaufils, S., Hartke, A. and Deutscher, J. (2007). "The phosphotransferase system of *Lactobacillus casei*: regulation of carbon metabolism and connection to cold shock response." J Mol Microbiol Biotechnol 12(1-2): 20-32.

Nakai, H., Svensson, B. and Ohtsubo, K. (2012). "Substrate recognition by two members of glycoside hydrolase family 32 involved in fructo-oligosaccharide metabolism in *Lactobacillus acidophilus* NCFM." Bulletin of the Faculty of Agriculture, Niigata University 64(2): 115 - 126.

Perez-Casal, J., Price, J. A., Maguin, E. and Scott, J. R. (1993). "An M protein with a single C repeat prevents phagocytosis of *Streptococcus pyogenes*: use of a temperature-sensitive shuttle vector to deliver homologous sequences to the chromosome of *S. pyogenes*." Mol Microbiol 8(5): 809-19.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res 29(9): e45.

Reid, S. J. and Abratt, V. R. (2005). "Sucrose utilisation in bacteria: genetic organisation and regulation." Appl Microbiol Biotechnol 67(3): 312-21.

Saulnier, D. M., Molenaar, D., de Vos, W. M., Gibson, G. R. and Kolida, S. (2007). "Identification of prebiotic fructooligosaccharide metabolism in *Lactobacillus plantarum* WCFS1 through microarrays." Appl Environ Microbiol 73(6): 1753-65.

Schwab, C. and Gänzle, M. G. (2006). "Effect of membrane lateral pressure on the expression of fructosyltransferases in *Lactobacillus reuteri*." Syst Appl Microbiol 29(2): 89-99.

Schwab, C., Walter, J., Tannock, G. W., Vogel, R. F. and Gänzle, M. G. (2007). "Sucrose utilization and impact of sucrose on glycosyltransferase expression in *Lactobacillus reuteri*." Syst Appl Microbiol 30(6): 433-43.

Siezen, R. J. and van Hylckama Vlieg, J. E. (2011). "Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer." Microb Cell Fact 10 Suppl 1: S3.

Sims, I. M., Frese, S. A., Walter, J., Loach, D., Wilson, M., Appleyard, K., Eason, J., Livingston, M., Baird, M., Cook, G. and Tannock, G. W. (2011). "Structure and functions of exopolysaccharide produced by gut commensal *Lactobacillus reuteri* 100-23." ISME J 5(7): 1115-24.

Stolz, P., Böcker, G., Hammes, W. P. and Vogel, R. F. (1995). "Utilization of electron acceptors by lactobacilli isolated from sourdough." Z Lebensm Unters Forsch 201: 91-96.

Stolz, P., Böcker, G., Vogel, R. F. and Hammes, W. P. (1993). "Utilization of maltose and glucose by lactobacilli isolated from sourdough." FEMS Microbiol Lett 109(2-3): 237-242.

Su, M. S., Oh, P. L., Walter, J. and Gänzle, M. G. (2012). "Intestinal origin of sourdough *Lactobacillus reuteri* isolates as revealed by phylogenetic, genetic, and physiological analysis." Appl Environ Microbiol 78(18): 6777-80.

Su, M. S., Schlicht, S. and Gänzle, M. G. (2011). "Contribution of glutamate decarboxylase in *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation." Microb Cell Fact 10 Suppl 1: S8.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). "MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods." Mol Biol Evol 28(10): 2731-9.

Teixeira, J. S., McNeill, V. and Gänzle, M. G. (2012). "Levansucrase and sucrose phoshorylase contribute to raffinose, stachyose, and verbascose metabolism by lactobacilli." Food Microbiol 31(2): 278-84.

Tieking, M., Ehrmann, M. A., Vogel, R. F. and Gänzle, M. G. (2005). "Molecular and functional characterization of a levansucrase from the sourdough isolate *Lactobacillus sanfranciscensis* TMW 1.392." Appl Microbiol Biotechnol 66(6): 655-63.

Trindade, M. I., Abratt, V. R. and Reid, S. J. (2003). "Induction of sucrose utilization genes from *Bifidobacterium lactis* by sucrose and raffinose." Appl Environ Microbiol 69(1): 24-32.

van den Broek, L. A., van Boxtel, E. L., Kievit, R. P., Verhoef, R., Beldman, G. and Voragen, A. G. (2004). "Physico-chemical and transglucosylation properties of recombinant sucrose phosphorylase from *Bifidobacterium adolescentis* DSM20083." Appl Microbiol Biotechnol 65(2): 219-27.

van Hijum, S. A., Kralj, S., Ozimek, L. K., Dijkhuizen, L. and van Geel-Schutten, I. G. (2006). "Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria." Microbiol Mol Biol Rev 70(1): 157-76.

Vogel, R. F., Knorr, R., Muller, M. R., Steudel, U., Gänzle, M. G. and Ehrmann, M. A. (1999). "Non-dairy lactic fermentations: the cereal world." Anton Van Leeuw J Microb 76(1-4): 403-11.

Walter, J. (2008). "Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research." Appl Environ Microbiol 74(16): 4985-96.

Walter, J., Schwab, C., Loach, D. M., Gänzle, M. G. and Tannock, G. W. (2008). "Glucosyltransferase A (GtfA) and inulosucrase (Inu) of *Lactobacillus reuteri* TMW1.106 contribute to cell aggregation, in vitro biofilm formation, and colonization of the mouse gastrointestinal tract." Microbiology 154(Pt 1): 72-80.

Wang, Y., Gänzle, M. G. and Schwab, C. (2010). "Exopolysaccharide synthesized by *Lactobacillus reuteri* decreases the ability of enterotoxigenic *Escherichia coli* to bind to porcine erythrocytes." Appl Environ Microbiol 76(14): 4863-6.

Yoon, M. Y. and Hwang, H. J. (2008). "Reduction of soybean oligosaccharides and properties of alpha-D-galactosidase from *Lactobacillus curvatus* R08 and *Leuconostoc mesenteroides* [corrected] JK55." Food Microbiol 25(6): 815-23.

CHAPTER FOUR: **GLUTAMINE, GLUTAMATE, AND ARGININE BASED ACID RESISTANCE IN *LACTOBACILLUS REUTERI*: REDUNDANT OR COMPLEMENTARY MECHANISMS?**

This chapter includes experimental work of Arisha Seeras, Alma Fernanda Sanchez-Maldonado, Dr. Chonggang Zhang, and Dr. Marcia Shu-Wei Su, performed under the supervision of Dr. Michael G. Gänzle. A version of this chapter will be submitted for publication in Applied and Environmental Microbiology.

CHAPTER 4

4.1. Introduction

Lactobacillus reuteri inhabits the intestinal tract of humans and animals; stable populations are found in the upper intestine of rodents, pigs, and poultry (Walter, 2008). *L. reuteri* also prevails in Type II sourdough fermentations that are carried out at elevated fermentation temperatures with long incubation times (Vogel *et al.*, 1999;). Both ecosystems impose acid stress. Type II sourdough fermentations expose *L. reuteri* to high concentrations of lactic acid at a pH of 3.5 for periods of several days (Vogel *et al.*, 1999;; Stromeck *et al.*, 2011). In the rodent forestomach, digesta remain for up to 20 h and are acidified to a pH of 3.5 to 4 by lactic metabolism (Ward and Coates, 1987; Gärtner, 2002). Subsequent gastric passage further acidifies digesta by gastric secretions of HCl (Ward and Coates, 1987).

Lactobacilli respond to acid stress by differential expression of stress proteins, and by up-regulation of metabolic pathways that contribute to pH homeostasis (Konings, 2002; De Angelis and Gobbetti, 2004). Figure 4-1 depicts metabolic activities known to contribute to acid resistance in *L. reuteri*. The multitude of complementary or redundant acid resistance mechanisms implies that *L. reuteri* is well equipped to withstand acidic environments. Indeed, metabolic activities of *L. reuteri* that increase acid resistance also contribute to its ecological fitness in sourdough and in the rodent intestine (Rollan *et al.*, 2003; Kaditzky *et al.*, 2008; Walter *et al.*, 2008; Su *et al.*, 2011).

The role of arginine and glutamate metabolism to bacterial acid resistance in general and specifically to acid resistance of *L. reuteri* is well understood (Cotter and Hill, 2003; Rollan *et al.*, 2003; Foster, 2004; Su *et al.*, 2011; Feehily

and KAratzas, 2013). However, while arginine metabolism is known to increase acid resistance by intracellular consumption of protons and the production of ATP (Konings, 2002), mechanisms of glutamine-glutamate system mediated acid resistance remain to be elucidated. The γ-carboxyl group of glutamate and GABA has a pK_A-value of 4.25, the proton balance of decarboxylation and transport is dependent on the intra- and the extracellular pH (Feehily and Karatzas, 2013). A contribution of glutamine deamidation to acid resistance was recently proposed in *Escherichia coli* but was not demonstrated experimentally in lactobacilli (Brown *et al.*, 2008; Lu *et al.*, 2013). Moreover, genetic determinants of glutamine conversion in lactobacilli remain to be elucidated.

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

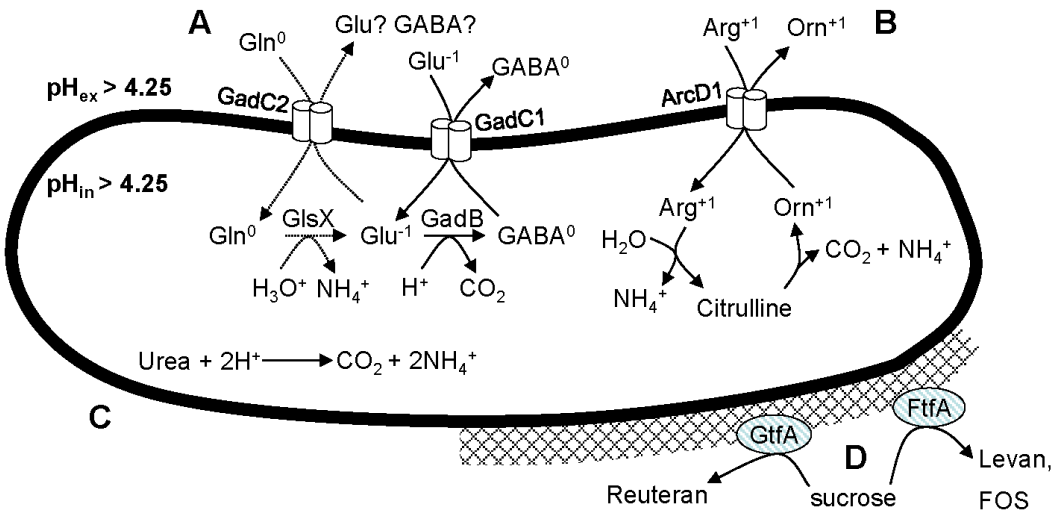


Figure 4-1. Metabolic pathways in *L. reuteri* contributing to acid resistance. Panel A. Glutamine converts to glutamate or GABA by one of three glutaminases (Gls1, Gls2, and Gls3) and strain-specific glutamate decarboxylase (GadB) improves survival at pH 2.5 (Su *et al.*, 2011). Panel B. The conversion of arginine to ornithine contributes to acid resistance in all strains of *L. reuteri* (Rollan *et al.*, 2003; De Angelis and Gobbetti, 2004). Panel C. Urease activity contributes to acid resistance in some rodent isolates of *L. reuteri* (Walter *et al.*, 2011; Wilson *et al.*, 2011). Panel D. The strain-specific formation of exopolysaccharides (reuteran, levan, or inulin) and oligosaccharides by glucansucrases (GtfA) or fructansucrases (FtfA) protects against membrane-active inhibitors and improve stationary phase survival (Schwab and Gänzle, 2006; Kaditzky *et al.*, 2008). GadC1 and GadC2, glutamate / GABA or glutamine / GABA antiporters. ArcD1, arginine-ornithine antiporter. GlsX, putative glutaminases Gls1, Gls2, and / or Gls3 in *L. reuteri*. Dotted line, hypothetical contribution to acid resistance or uncharacterized gene functions. Ion charge assigned to molecules reflect intracellular and extracellular pH-values higher than 4.25.

4.2. Materials and methods

**4.2.1. Media, strains and growth conditions*

Lactobacillus reuteri 100-23 and 100-23Δ*gadB* (Su *et al.*, 2011) were grown in modified MRS medium (mMRS) (Ganzle et al., 1998) at 37°C. Ten mmol L^{-1} of glutamine were added where indicated. Frozen stock cultures of *L. reuteri* were inoculated on mMRS agar plates, single colonies were inoculated into one mL mMRS medium, subcultured with 1% inoculum in mMRS medium, and grown to the exponential phase (5 h of incubation, OD_{600} = 0.5; pH = 4.5) or the late stationary phase (48 h of incubation, OD_{600} = 1.6; pH = 3.5).

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

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

To determine the effect of acid stress on *gls1*, *gls2, gls3, adi*, and *gadB* expression in *L. reuteri* 100-23, the strain was grown to the exponential phase. Cells were centrifuged, and resuspended in mMRS acidified to pH 3.5 with HCl.

Asterisks (*) indicate contributions by Arisha Seeras, Alma Fernanda Sanchez-Maldonado, Dr. Chonggang Zhang, and Dr. Marcia Shu-Wei Su.

Cells were centrifuged, and resuspended in mMRS acidified to pH 3.5 with HCl. After 40 min of incubation in mMRS (pH 3.5) at 37°C, cultures were mixed with RNAprotect Bacteria Reagent and cell pellets were harvested and stored at -80 ºC. Sourdoughs were prepared with 1 mL of an overnight culture of *L. reuteri* 100-23, 10 mL sterile tap water, and 10 g whole wheat flour. Dough was incubated at 37 ºC and samples were taken after 5 h (exponentially growing cells) or 48 h (stationary phase of growth). Aliquots of 0.5 g sourdough were mixed with 3 mL of RNAprotect Bacteria Reagent (Qiagen), incubated at room temperature for 10 min, and the solids were removed by centrifugation at 400 *g* for 10 min. Cells in the supernatant were harvested by centrifugation and the cell pellets were stored at -80ºC prior to RNA isolation.

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

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

Specific primers targeting *gls1, gls2, gls3, adi,* and *gadB* (Table 4-1) were used for qPCR amplification, which was performed using the QuantiFast SYBR green master mixture (Qiagen) in a 7500 Fast Real Time-PCR System (Applied Biosystems, the USA). Primers were designed based on the genome sequence of *L. reuteri* 100-23 (GenBank Accession number AAPZ02000002.1). To verify the specificity of the primers, qPCR reactions were carried out with chromosomal

DNA as template for subsequent determination of the size and melting point of the amplicons. Chromosomal DNA isolated from *L. reuteri* 100-23 and DNase-treated RNA samples were used as positive and negative controls, respectively, in all RT-qPCR reactions. Normalized gene expression ratios were calculated according to Pfaffl (2001) using the gene *pho* coding for phosphoketolase in *L. reuteri* as reference gene, and exponentially growing cultures of *L. reuteri* 100-23 in mMRS as reference condition (N_0). The efficiencies of the PCR reactions were determined in PCR reactions with serial 10-fold dilutions of chromosomal DNA of *L. reuteri* 100-23 as template. The efficiencies (*E*) of PCR reactions targeting *pho, gls1, gls2, gls3, adi*, and *gadB* were 1.90, 1.90, 1.91, 1.89, 2.00, and 2.00, respectively. Results are reported as means ± standard deviation of duplicate independent experiments analyzed in duplicate.

***Table 4-1.** List of primers used in RT-qPCR

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

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

To determine the relevance of different acid resistance mechanisms of *L. reuteri* at different pH values, the survival of *L. reuteri* 100-23 and

100-23Δ*gadB* was compared after acid stress in 100 mmol L^{-1} lactate buffer (pH 3.5) and in 100 mmol L^{-1} phosphate buffer (pH 2.5). Buffers were supplemented with 20 mmol L^{-1} arginine, glutamine, or glutamate as indicated, and the pH was re-adjusted to pH 3.5 or 2.5 after the addition of amino acids. Overnight grown cells were harvested and resuspended in lactate or phosphate buffers. Based on previous investigations of acid resistance of *L. reuteri* 100-23 (Su *et al.*, 2011), cells were incubated at 37ºC for 24 h in the pH 3.5 buffer, or at 37ºC for 10 h in the pH 2.5 buffer prior to the enumeration of viable cell counts by plating. Results are expressed as mean value ± standard deviation of four independent experiments. Statistical analysis was performed using Student's *t*-test.

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

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

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

The effect of amino acids on transmembrane proton potential was determined by quantifying internal pH with a conjugated fluorescent pH probe 5(6)-carboxyfluorescein diacetate succinimidyl ester (cFDASE; Sigma). An assay

based on the method proposed by Breeuwer *et al.* (1996) was used to measure the pH_{in} of *L. reuteri* 100-23 and 100-23Δ*gadB*. Modifications of the method were required to maintain *L. reuteri* metabolically active and with a measurable transmembrane potential throughout the staining protocol (Gänzle and Vogel, 2003). In brief, cells were harvested by centrifugation from overnight cultures in mMRS, washed and resuspended in 50 mmol/L citrate phosphate buffer (pH 5.0). Subsequently, the cells were incubated in the presence of 10 μmol/L cFDASE at 37°C for 15 min. After labeling with cFDASE, cells were collected by centrifugation and resuspended to an OD_{600} 10.0 in 50 mmol/L citrate phosphate buffer (pH 5.0) containing 10 mmol/L of maltose and fructose, 0.4 mmol/L $MgSO_4$, and 0.3 mmol/L $MnSO_4$ to eliminate non-conjugated probe. The cells were subsequently diluted to an OD_{600} 1.0 in 20 mmol/L citrate phosphate buffer (pH 4.0), 100 mmol/L lactate buffer (pH 4.0), or 100 mmol/L acetate buffer (pH 5.0) supplemented with 20 mmol/L arginine, glutamine, or glutamate as indicated. Two different buffers were used at a pH of 4.0 in order to investigate whether the choice of the buffer affects amino acid dependent systems for pH homeostasis. The cultures and all buffers were maintained at 37°C to maintain the cells metabolically active. Centrifuges were maintained at ambient temperature and centrifugation steps were limited to 3 min.

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

monochromator between both wavelengths. The emission wavelength was 525 nm, and the excitation and emission slit width was 5 nm. Calibration curves for *L. reuteri* and its mutant 100-23Δ*gadB* were determined in 50 mmol/L citrate phosphate buffers with pH values ranging from 4 to 8. The pH_{in} and pH_{out} were equilibrated by addition of nigericin (Sigma) and valinomycin (Sigma) to a final concentration of 5 μmol/L, and the ratios were determined as described previously (Breeuwer *et al.*, 1996). Calibration curves were established for each batch of labeled cells. Data presented are means ± standard deviations from three independent experiments.

4.2.8. Monitoring of the transmembrane electrical potential (ΔΨ)

Changes in transmembrane potassium potential in *L. reuteri* 100-23 and its mutant 100-23Δ*gadB* were monitored using the fluorescent probe 3,3'-dipropylthiacarbocyanine [$DiSC_3(5)$; Invitrogen] based on a previously described method (Gänzle and Vogel, 2003). The harvested cells were washed once with 20 mmol/L citrate phosphate buffer (pH 6.5), 20 mmol/L citrate phosphate buffer (pH 4.0), or 100 mmol/L lactate buffer (pH 4.0), containing 10 mmol/L of maltose and fructose each as carbon source and 0.4 mmol/L $MgSO_4$ and 0.3 mmol/L $MnSO_4$. After centrifugation, the cells were resuspended to an OD_{600} 0.5 in the same buffers supplemented with 20 mmol/L of arginine, or glutamine, or glutamate as indicated. Measurements were carried out at t=0, t=12 hours, and t=24 hours after incubation at 37°C. The cells were transferred to a microtiter plate containing $DiSC_3(5)$ (final concentration of 5 μmol/L). The cells

were incubated for about 10 minutes to equilibrate the internal and external dye concentrations, followed by the addition of the proton ionophore nigericin (1 μmol/L) and the potassium ionophore valinomycin (1 μmol/L). Fluorescence measurements were performed using a microtiter plate reader (Varioskan Flash) with excitation and emission wavelengths of 643 and 666 nm, respectively (slit widths of 5 nm). The data shown are representative of at least three experiments that gave similar results.

4.3. Results

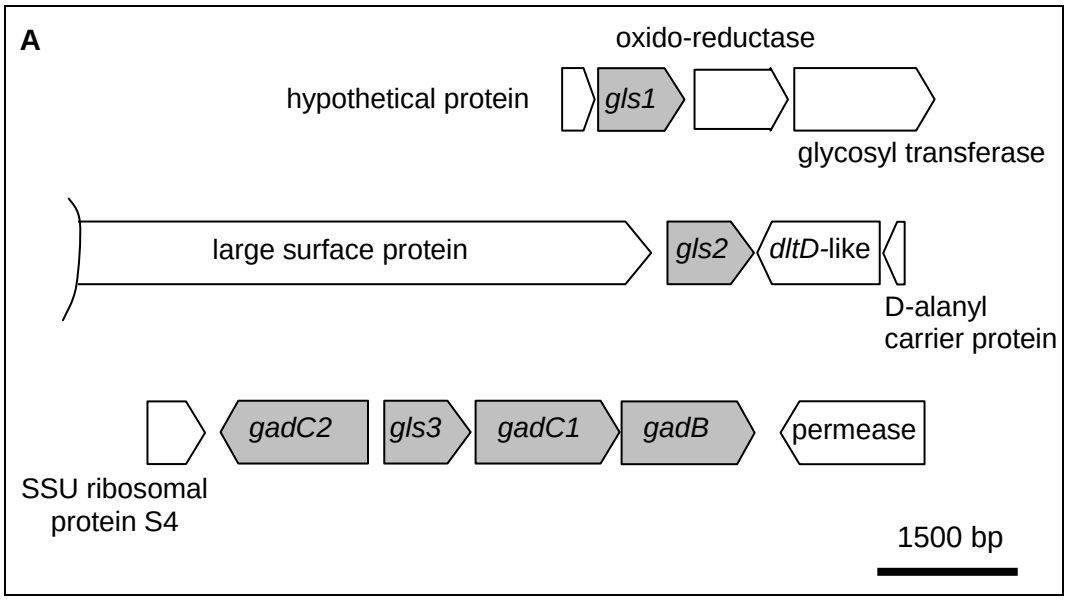
*4.3.1. Identification of glutaminases in the genome of L. reuteri 100-23

To identify the genetic determinants of glutamine deamidation in *L. reuteri* 100-23, putative glutaminases were identified in the genome of *L. reuteri* 100-23 (Figure 4-2A). The putative glutaminase gene *gls3* is located adjacent to the glutamate decarboxylase *gadB* (Su *et al.*, 2011) and the putative glutamate/GABA antiporters *gadC1* and *gadC2*. The genes *gls1* and *gls2* are not located in the vicinity of other genes involved in glutamine metabolism or transport (Figure 4-2A). The protein sequences of Gls1, Gls2, and Gls3 in *L. reuteri* 100-23 are 44% to 66% identical to each other and 28% to 38% identical to glutaminases of *E. coli* W3110 (YbaS and YneH) and *B. subtilis* ATCC 23857D-5 (YbgJ and YlaM) (Brown *et al.*, 2008, Figure 4-2B). Serine-dependent β-lactamases, penicillin-binding proteins, and glutaminases have been assigned into a large protein family; alignment of the glutaminase protein sequences confirmed that the conserved catalytic residues S-X–X-K (S60-K63 in

YbaS, β-lactamase motif I), the β-lactamase motif III (K259-S-G261 in YbaS), and the incomplete β-lactamase motif II (S160 in YbaS) were also conserved in the three glutaminases of *L. reuteri* (Brown *et al.*, 2008, Figure 4-2C, and data not shown). Moreover, of the 40 amino acid residues that are conserved in 8 eukaryotic and prokaryotic glutaminases (Brown *et al.*, 2008), 37, 30, and 36 residues were also conserved in *gls1*, *gls2*, and *gls3*, respectively. PCR analysis of cDNA libraries demonstrated that *gls1*, *gls2*, and *gls3* are expressed in *L. reuteri* (Table 4-2 and data not shown). Protein homologies, the presence of conserved catalytic residues, and confirmation of gene expression indicate that *gls1*, *gls2*, and *gls3* of *L. reuteri* 100-23 code for three functional glutaminases.

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

The expression of amino-acid based mechanisms of acid resistance was quantified in stationary phase cells growing in mMRS, after exposure of exponentially growing cells to acid stress, and in mMRS supplemented with glutamine (Table 4-2). Acid stress induced by the stationary phase of growth, or by exposing exponentially growing cells to pH 3.5, consistently resulted in over-expression of *gls3* and *gadB* (Table 4-2). Supplementation of mMRS with 10 mmol L^{-1} glutamine resulted only in a two-fold increase of *gls3* expression after 48 h of inoculation. Expression levels of *gls1* and *gls2* were not influenced by culture conditions in mMRS (Table 4-2). Surprisingly, expression levels of *adi* were essentially unchanged after acid stress, and reduced in stationary phase cells.



B

	YbgJ	YlaM	YbaS	YneH	Gls1	Gls2	Gls3
Gls1	35%	38%	36%	33%	/	44%	66%
Gls2	32%	32%	28%	30%	/	-	45%
Gls3	36%	38%	36%	34%	/	/	-

C

```
      24          57            117   257              276
YbaS  GQNADYIPFL GDSDYRFALESISKV NAGA PGKSGVGGGILAVVP IAAFSPPLDEDGNSVRG
YneH  GKVADYIPAL GDAQERFSIQSISKV NAGA PAKSGVGGGIVAIVP IAVWSPELDDAGNSLAG
YbgJ  GQSANYIPAL GDWNVSFTMQSISKV NAGA PAKSGVSGGIMALVP IGIYGPAIDEYGNSLTG
YlaM  GEVASYIPAL GDVEKTFTLQSISKV NAGA PAKSGVSGGIMGISP IGIFGPALDEKGNSIAG
Gls1  GQVATYIPAL GASQVRFAIESVSKV NSGA PTKSGVGGGLVSAAP IGIFSPALDHAGNSVAG
Gls2  GKVANYIPAL GMAGTRFAIESIAKV NSSA PIKSGTGGGLLACAP IGIFSPALDQHGNSLAG
Gls3  GKVANYIPAL GNADVRFAIESISKV NAGA PTKSGVGGGLMSAAP IGIFSPPLDNAGNSVAG
      *  * *** * *     *   *  ** **** * ** * *      *      *     ***  *
```

***Figure 4-2.** Genes coding for glutaminases in *L. reuteri* 100-23. Panel A. Representation of genetic loci coding for glutaminases in *L. reuteri* 100-23. Sequences and annotations are retrieved from GenBank (Accession number AAPZ02000002.1) and (Frese *et al.*, 2011; Su *et al.*, 2011). IMG Gene Object IDs of *gls1*, *gls2*, and *gls3* are 639134920, 639133703, and 639134641, respectively. Genes coding for glutamine transport or metabolism are shaded in grey. Panel B. Protein identity (%) of glutaminases in *L. reuteri* to the glutaminases YbgJ and YlaM in *B. subtilis*, and YbaS and YneH in *E. coli*. Panel C. Alignment of conserved domains in glutaminases in *L. reuteri* to glutaminases in *E. coli* (YbaS and YneH) and *B. subtilis* (YbgJ and YlaM) (Brown *et al.*, 2008). Numbers correspond to the YbaS sequence; highly conserved amino acids (Brown *et al.*, 2008) are marked by asterisks and deviating amino acids in Gls1, Gls2, or Gls3 are underlined.

The amino acid availability and the organic acid production in cereal substrates differ substantially from laboratory media; gene expression was therefore also quantified in wheat sourdough fermentations with *L. reuteri* 100-23 (Table 4-2). Amino acid analysis after 48 h of fermentation confirmed that arginine conversion to ornithine as well as glutamine conversion to γ-aminobutyrate occurred and were not limited by substrate depletion (Stromeck *et al.*, 2011, and data not shown). Stationary phase cultures in wheat sourdough over-expressed *gls3* and *gadB* more than 100-fold; additionally *gls2* was over-expressed (Table 4-2). Expression levels of *adi* were reduced in exponentially growing cells in sourdough.

***Table 4-2.** Relative quantification of the expression of the glutaminases *gls1, gls2,* and *gls3*, glutamate decarboxylase *gadB*, and arginine deiminase *adi* in *L. reuteri* 100-23. Gene expression was quantified in exponentially growing (5 h) or stationary phase (48 h) cultures in mMRS or mMRS-glutamine, after acid shock in mMRS, and during growth in sourdough. Gene expression in samples (N) was quantified relative to exponentially growing cells in mMRS medium (N_0); *pho* coding for phosphoketolase was used as reference gene. Results were calculated as N/N_0 and are reported as mean ± standard deviation of duplicate experiments analyzed in duplicate.

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

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

*4.3.3. Expression of glutaminases, glutamate decarboxylase, and arginine deiminase in L. reuteri 100-23Δ*gadB

To determine whether disruption of one acid resistance mechanism is compensated by over-expression of other acid resistance genes, gene expression levels in *L. reuteri* 100-23Δ*gadB* were quantified relative to the wild type strain *L. reuteri* 100-23 growing cells in mMRS medium (N_0) (data not shown). Deletion of *gadB* resulted in over-expression of *adi* (4.5 ± 1.2) and *gls3* (28.4 ± 10.4) at 48 h of cultivation.

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

The survival of *L. reuteri* in acidic conditions was determined in lactate buffer (pH 3.5) to mimic conditions in the forestomach of rodents and in sourdoughs (Gänzle *et al.*, 1998; Gärtner, 2002; Stromeck *et al.*, 2011), and in phosphate buffer (pH 2.5) to imitate gastric transit metabolism (Ward and Coates, 1987; Arnold and Kaspar, 1995). Lactate and phosphate buffers were supplemented with amino acids to evaluate their contribution to survival (Figure 4-3).

Glutamine and glutamate but not arginine improved survival at pH 2.5 (Figure 4-3A). To determine whether the protective effect of glutamine on the survival of *L. reuteri* 100-23 in acidic condition at pH 2.5 is mediated by glutamine deamidation or by glutamate decarboxylation (Figure 4-1), the experiment was also carried out using *L. reuteri* 100-23Δ*gadB*. In keeping with prior observations (Su *et al.*, 2011), disruption of *gadB* eliminated the protective

effect of glutamate on the survival of *L. reuteri* 100-23Δ*gadB*. However, glutamine remained protective for *L. reuteri* 100-23Δ*gadB*, demonstrating that glutamine deamidation protects against acid stress in the absence of glutamate decarboxylation (Figure 4-3B). Arginine provided no protection at a pH of 2.5; however, survival at pH 3.5 was improved by arginine (Figure 4-3C). In contrast, glutamine or glutamate had no significant protective effect at pH 3.5, indicating that the effect of amino acid conversions on acid resistance is dependent on the ambient pH.

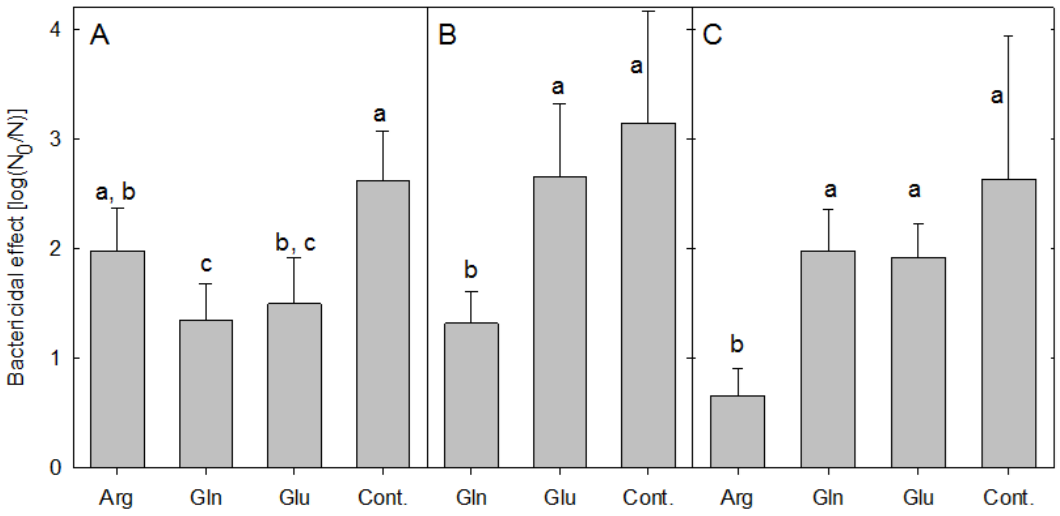


***Figure 4-3**. Survival of *L. reuteri* 100-23 (Panels A and C) and *L. reuteri* 100-23Δ*gadB* (Panel B) in acid stress. Cells were incubated in phosphate buffer at pH 2.5 for 10 h (Panels A and B) or in lactate buffer at pH 3.5 for 24 h (Panel C). Control treatments were carried out in phosphate or lactate buffers (cont.); arginine, glutamine or glutamate were added to a final concentration of 20 mmol L^{-1} as indicated. Data are shown as means ± standard deviation of four independent experiments. Data obtained for the same strain at the same pH that do not share a common superscript are significantly different ($p < 0.05$).

4.3.5. Effects of amino acid metabolism on the transmembrane potential (ΔpH and ΔΨ)

The divergent effects of arginine and glutamate or glutamine on survival at acidic conditions imply that arginine- and glutamate-dependent acid resistance is dependent on different mechanisms. The role of amino acid-based acid resistance in *L. reuteri* 100-23 was evaluated by measuring changes in the intracellular pH and the dissipation of transmembrane potassium potential of *L. reuteri.* To determine if the protective effect of glutamine and glutamate on the survival of *L. reuteri* 100-23 under acidic condition is mediated by glutamine deamidation or by glutamate decarboxylation, the experiments were also carried out with *L. reuteri* 100-23Δ*gadB.*

The intracellular pH was measured by the cFDASE method. This fluorescent dye exhibits a pK_A value of 6.5, which is suitable for the determination of pH_{in}-values in the expected range of 5.0 – 7.5. However, experiments performed at conditions that kill *L. reuteri* (pH 2.5 or pH 3.5) indicated that pH_{in} decreased rapidly to values below 4.0, i.e. the limit of pH range that can be determined with cFDASE (data not shown). Therefore, the pH_{in} was determined at an external pH 4.0 (citrate phosphate and lactate buffers) and 5.0 (acetate buffer).

L. reuteri 100-23 decreased its pH_{in} values as a function of the pH_{ex} (Figure 4-4). Results obtained in presence of arginine confirmed that the arginine deiminase (ADI) pathway consumes intracellular protons, and causes the alkalization of the fermentation substrate. An increased intracellular pH was observed in all of the

three buffers employed (Figure 4-4). *L. reuteri* 100-23Δ*gadB* showed a higher increase of the pH_{in} by arginine conversion. Glutamate conversion significantly increased the pH_{in} in citrate-phosphate buffer, pH 4.0; this effect was not observed

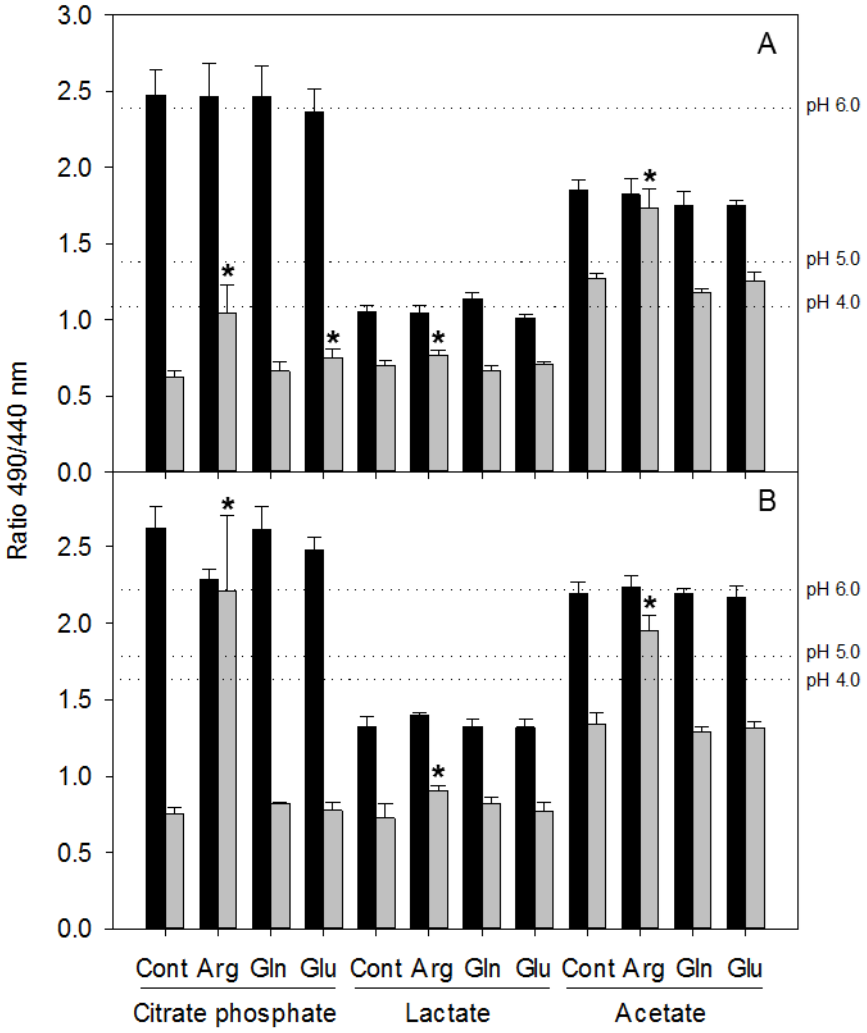


Figure 4-4. Effect of arginine, glutamine, and glutamate on the pH_{in} of *L. reuteri* 100-23 (Panel A) and its isogenic mutant *L. reuteri* 100-23Δ*gadB* (Panel B) after 0 h (black columns) and 24 h (grey columns) of incubation at 37°C in citrate phosphate (pH 4.0), lactate (pH 4.0), and acetate (pH 5.0) buffers. Ratio of cFSE in stained cells of *L. reuteri* 100-23 at excitations of 490 and 440 nm, and emission wavelengths of 525 nm. Controls line (dashed lines) represent the ratio where pH_{in} and pH_{ex} were equilibrated by incubation with nigericin (1 µM) and valinomycin (1 µM). Data are shown as means ± standard deviations of three independent experiments. Significant differences ($p < 0.001$) between the presence and absence of amino acids were determined by Student`s *t*-test and are indicated by asterisks.

with glutamine or in other buffer systems. *L. reuteri* 100-23 and 100-23Δ*gadB* remained viable during incubation at pH 4.0 despite the apparent lack of a transmembrane proton potential (Figure 4-4 and data not shown).

The contribution of the transmembrane potassium potential (ΔΨ) to amino acid-based acid resistance was investigated with the cationic fluorescent probe $DiSC_3(5)$. During measurements in the presence or absence (control) of amino acids, the proton motive force (pmf) was manipulated with the proton-ionophore nigericin and the potassium ionophore valinomycin. In citrate phosphate buffer (pH 6.5), nigericin addition increased the membrane potassium potential because the loss of the ΔpH is compensated by an increase in the ΔΨ, as evidenced by decreased fluorescence intensity (Figure 4-5). Valinomycin addition abolished the potassium potential (Figure 4-5). Monitoring of the changes of membrane potential in the presence of amino acids at this condition indicated that the ΔpH and the ΔΨ were higher in *L. reuteri* 100-23 and 100-23Δ*gadB* incubated with arginine when compared to the control.

To further confirm the involvement of ΔΨ in the amino acid-based acid resistance, the monitoring was done at pH_{ex} 4.0. Protonophores are expected to specifically increase the electric proton conductance of the membrane; however, at pH_{ex} 4.0, the ionophore nigericin aggregates in a trimer and also transfers K^+ across the hydrophobic membrane (Toro *et al.*, 1987). In keeping with the lack of a transmembrane ΔpH in *L. reuteri* at an external pH of 4.0, the addition of nigericin did not decrease $DiSC_3(5)$ fluorescence because cells compensated for the loss of ΔpH with an increase in the potassium gradient, but mediated a slow

dissipation of the transmembrane potassium gradient (Figure 4-5). However, addition of valinomycin resulted in a rapid dissipation of the transmembrane potassium gradient, and a rapid increase in $DiSC_3(5)$ fluorescence, in all cultures. At a pH_{ex} of 4.0, the addition of valinomycin showed a higher increase in probe fluorescence when glutamate was present, indicating a higher in transmembrane potassium potential in presence of glutamate. This effect was not observed with *L. reuteri* 100-23Δ*gadB*, demonstrating that the effect is attributable to glutamate decarboxylation.

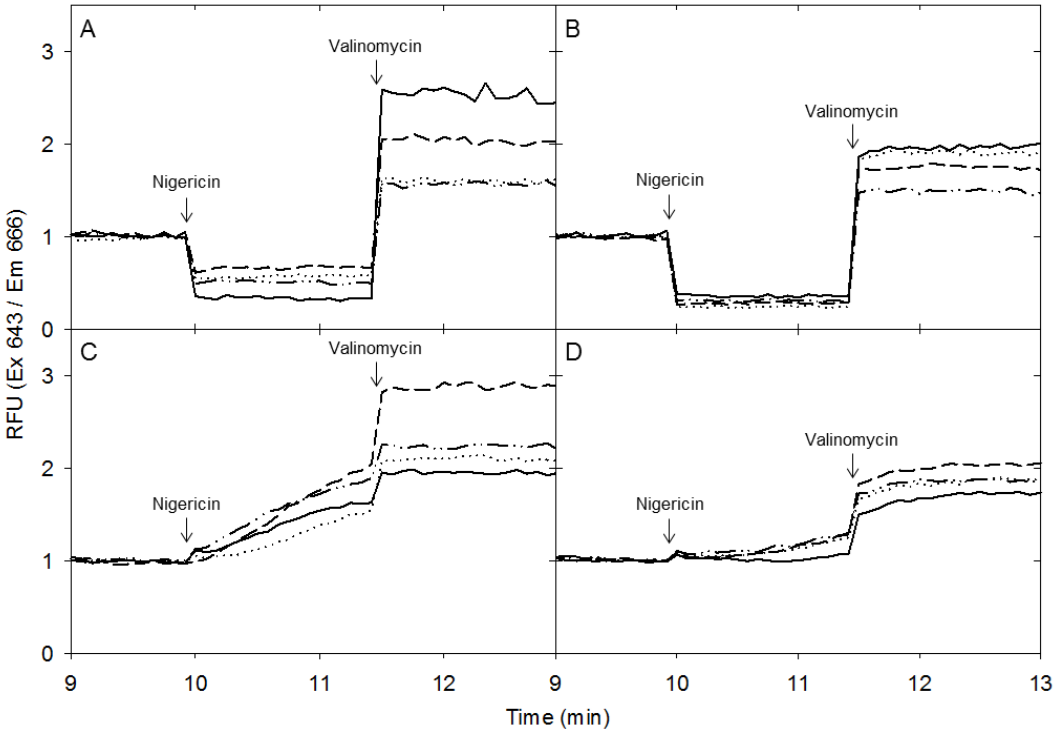


Figure 4-5. Effect of arginine (—), glutamine (·····), and glutamate (– –) on the membrane potential of *L. reuteri* 100-23 (Panels A and C) and its isogenic mutant *L. reuteri* 100-23Δ*gadB* (Panels B and D) after 12 hours of incubation in citrate-phosphate (pH 6.5, Panels A md B) and lactate (pH 4.0, Panels C and D) buffers at 37°C. Fluorescent traces of $DiSC_3(5)$ in stained cells of *L. reuteri* 100-23 were determined at excitation and emission wavelengths of 643 and 666 nm, respectively. Nigericin and valinomycin were added to a final concentration of 1 μmol/L at the time points indicated by the arrows to check the dissipation of the ΔpH and ΔΨ, respectively. Control, ·–·. RFU, relative fluorescence units. The results are representative for three independent experiments.

4.4. Discussion

Past studies on the role of glutamate in acid resistance of *L. reuteri* indicated that glutamate conversion is dependent on glutaminases to convert the abundant amino acid glutamine to glutamate (Su *et al.*, 2011). The present study demonstrated that glutamine deamidation improves survival of *L. reuteri* in pH 2.5 conditions independent of glutamate decarboxylation. Bioinformatic analyses of glutaminase genes in *L. reuteri* 100-23, and the relative quantification of their expression in indicated that this strain harbors three glutaminase genes. Glutamine conversion in *L. reuteri* 100-23 is predominantly attributable to the proteins encoded by the *gls3-gadB* operon.

Glutamine, glutamate or arginine conversions consume intracellular protons and thus contribute to pH increase acid resistance (Cotter and Hill, 2003; Foster, 2004; Su *et al.*, 2011; Lu *et al.*, 2013). Arginine is decarboxylated to agmatine by *E. coli* and related organisms (Foster, 2004; Richard and Foster, 2004); *Listeria monocytogenes* and lactic acid bacteria convert arginine to ornithine via the arginine-deiminase pathway (ADI) (Konings, 2002; Rollan *et al.*, 2003; De Angelis and Gobbetti, 2004). Glutamine improved acid resistance in *E. coli* was recently demonstrated (Lu *et al.*, 2013). The amino acid composition of cereal proteins (Wieser, 2007), and the organization of genes coding for glutamine and glutamate metabolism imply a role of glutamine in acid resistance in *L. reuteri* 100-23 (Su *et al.*, 2011). This study demonstrated that glutamine deamidation by *L. reuteri* provides a level of protection that is comparable to glutamate decarboxylation. *L. reuteri* 100-23 thus maintains three metabolic

pathways for amino acid-mediated acid resistance; urease activity and fructan formation additionally improve acid resistance (Figure 4-1 and 4-6). Several lines of evidence suggest that these pathways are partially redundant. (i) Strains of *L. reuteri* generally converts arginine and glutamine whereas glutamate decarboxylation in *L. reuteri* is strain-specific (Rollan *et al.*, 2003; Vermeulen *et al.*, 2007; Stromeck *et al.*, 2011). (ii) Glutamine deamidation and glutamate decarboxylation provide comparable levels of protection at pH 2.5. (iii) *L. reuteri* 100-23 harbours three different glutaminases. The coordinated over-expression of *gls3* and *gadB*, both located on the same operon, indicates that these genes are mainly responsible for glutamine conversion in *L. reuteri* 100-23. Disruption of glutamate decarboxylase in *L. reuteri* 100-23Δ*gadB* was compensated by over-expression of *adi* and *gls3.* This result conforms with the conditional over-expression of the ADI pathway in *Lactococcus lactis* if glutamate was absent (Mazzoli *et al.*, 2010). This redundancy in acid resistance mechanisms is remarkable for an organism that has adapted by reduction of genome size (Frese *et al.*, 2011; Walter *et al.*, 2011).

Mechanisms of acid resistance are complementary with respect to the use of substrates. Urea is available in mammalian gastric environments; arginine and glutamine are released by proteolysis from cereal proteins (Thiele *et al.*, 2002; Wieser, 2007). Moreover, the contribution of amino acid conversions to pH homeostasis is dependent on both the intracellular and the extracellular pH (Konings, 2002; Ma *et al.*, 2013; Tsai *et al*., 2013). The majority of studies related to glutamate- or arginine-mediated bacterial acid resistance simulated gastric

survival at pH 2 – 2.5(Arnold and Kaspar, 1995; Hersh *et al.*, 1996;; Diez-Gonzalez *et al.*, 1998;Cotter and Hill, 2003; Foster, 2004; Karatzas *et al.*, 2010; Feehily and Karatzas, 2013). At a pH of 2, glutamate decarboxylation but not arginine decarboxylation improved survival of *E. coli* (Hersh *et al.*, 1996). This study compared the survival of *L. reuteri* at pH 2.5 in a phosphate buffered system with the survival at pH 3.5 in a lactate-buffered system, thusdifferentiating between survival in a gastric environment, and survival under conditions simulating the rodent forestomach or long-time sourdough fermentations (Ward and Coates, 1987; Gänzle *et al.*, 1998; Gärtner, 2002). Arginine protected *L. reuteri* at pH 3.5 while glutamine or glutamate protected *L. reuteri* at pH 2.5. This result demonstrated that acid resistance mechanisms are complementary with respect to their function at different conditions.

Remarkably, *L. reuteri* did not maintain a high intracellular pH at acidic pH_{ex}. Likewise, beer spoiling *L. brevis* survives at low pH values without maintaining a large transmembrane ΔpH (Schurr *et al.*, 2013). The transmembrane ΔpH was lower at high external lactate concentrations, likely because pH homeostasis by H^+/lactate symport is less effective at high external lactate concentrations (Konings and Otto, 1983). The ability of lactobacilli to survive with a low pH_{in} contrasts observations in *E. coli* where acidification of the cytoplasm below pH 5.0 compromises survival (Foster, 2004; Ma *et al.*, 2013).

Arginine conversion by *L. reuteri* consumes two intracellular protons; the production of ATP enables the additional extrusion of protons by F_0F_1-ATPase (Konings, 2002; Cotter and Hill, 2003). This study confirmed cytoplasmic

alkalization as a result of arginine conversion in *L. reuteri*. The lack of protective effect of arginine at pH 4.0 or less may relate to the low activity of enzymes in the ADI pathway at pH of 4.0 (De Angelis *et al.*, 2002). Arginine conversion thus provides protection predominantly at moderate levels of acidity.

The effect of glutamate conversion on the pmf depends on the extra- and intracellular pH because the consumption of protons and the polarization of the membrane differ depending on the species of glutamate (Glu^{-1}, Glu^{-0}, or Glu^{+1}) and GABA ($GABA^{0}$ or $GABA^{+1}$) which are transported. At pH values of less than 4.0, the glutamate α-carboxyl group remains charged but the γ-carboxyl group (pK_A = 4.25) is mostly protonated. Decarboxylation of glutamate eliminates one cytoplasmic proton; Glu^{0}/$GABA^{+1}$ antiport additionally exports one charge (Ma *et al.*, 2013, Figure 4-6). We observed an increase of the cytoplasmic pH as well as polarization of the membrane by glutamate decarboxylation in *L. reuteri*, suggesting comparable mechanisms in *L. reuteri*.

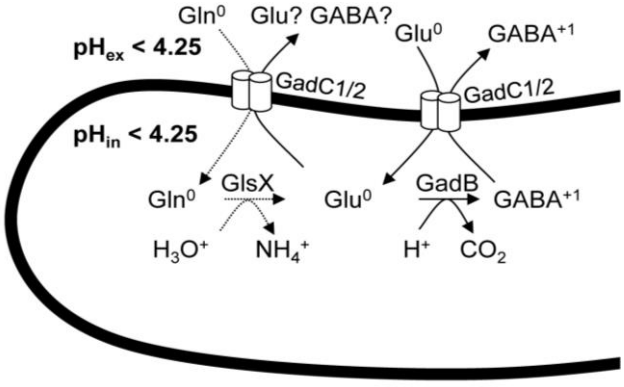


Figure 4-6. Metabolic pathway for glutamine-glutamate based acid resistance in *L. reuteri*. Follow uptake of glutamine by unknown mechanisms, glutaminase catalyze deamidation to glutamate and ammonia. Under acidic conditions ($pH_{ex} \leq 4.0$), the glutamate α-carboxyl group remains charged but the γ-carboxyl group with a pK_A of 4.25 is mostly protonated if the pH_{in} is less than 4.25. Decarboxylation of glutamate eliminates one cytoplasmic proton; Glu^{0}/$GABA^{+1}$ antiport additionally exports one charge.

Glutamate or glutamine conversion affected survival or pmf in *L. reuteri* only at pH 4.0 or less, matching conditions at which the *gls3-gadB* operon is highly expressed and, conditions where Glu^{0} and $GABA^{+1}$ are predominant species. However, the increase of the cytoplasmic pH was observed only in citrate-phosphate buffer and it was small when compared to arginine, indicating that the membrane polarization may be more relevant for survival of *L. reuteri*. The deamidation of glutamine is also predicted to consume one proton (Figure 4-6, Lu et al., 2013) but this study failed to detect cytoplasmic alkalinisation. In *E. coli*, GadC also transports glutamine (Lu et al., 2013) but the specificity of *gadC1* and *gadC2* in *L. reuteri* remains unknown.

Genetic and physiological analyses of *L. reuteri* show that different lineages of *L. reuteri* have evolved with their hosts (Frese *et al.*, 2011). Differences in genome content between host-adapted lineage also relate to acid resistance (Frese *et al.*, 2011). Rodent isolates but not human isolates of *L. reuteri* exhibit urease activity and the *gls3-gadB* operon is found only in rodent isolates (Frese *et al.*, 2011). The substrates urea and glutamine are abundant in gastric environments; urease as well as *gls3* and *gadB* during gastric transit (Wilson *et al.*, 2011). Genetic differences between rodent and human-adapted strains of *L. reuteri* may reflect that residents of the rodent forestomach but not human colonic microbiota are exposed to gastric acidity.

Multiple amino acid-based acid resistance systems are also relevant for the prevalence of *L. reuteri* in the Type II sourdough ecosystems (Vogel *et al.*, 1999; Su *et al.*, 2011). Glutamate decarboxylation increased the competitiveness of

L. reuteri 100-23 in sourdough (Su *et al.*, 2011). This study provided evidence that arginine is more effective than glutamine / glutamate conversions at conditions prevailing during long-term sourdough fermentations. It is noteworthy that metabolic pathways in *L. reuteri* that contribute to acid resistance (Figure 4-1) also improve the bread quality (Thiele *et al.*, 2002; Su *et al.*, 2011; Galle and Arendt, 2013). The analysis of genetic determinants of glutamine conversion in lactobacilli thus facilitates the selection of starter cultures for bread quality improvements.

4.5. References

Arnold, K. W. and Kaspar, C. W. (1995). "Starvation- and stationary-phase-induced acid tolerance in *Escherichia coli* O157:H7." Appl Environ Microbiol 61(5): 2037-9.

Breeuwer, P., Drocourt, J., Rombouts, F. M. and Abee, T. (1996). "A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester." Appl Environ Microbiol 62(1): 178-83.

Brown, G., Singer, A., Proudfoot, M., Skarina, T., Kim, Y., Chang, C., Dementieva, I., Kuznetsova, E., Gonzalez, C. F., Joachimiak, A., Savchenko, A. and Yakunin, A. F. (2008). "Functional and structural characterization of four glutaminases from *Escherichia coli* and *Bacillus subtilis*." Biochemistry 47(21): 5724-35.

Copeland, A. L. S., Lapidus, A., Barry, K., Detter, J. C., Rio, T. G. d., Hammon, N., Israni, S., Dalin, E., Tice, H., Pitluck, S., Sun, H., Schmutz, J., Larimer, F., Land, M., Hauser, L., Walter, J., Heng, N. C. K., Tannock, G. W. and Richardson, P. (2008). "Permanent draft sequence of *Lactobacillus reuteri 100-23*." US DOE Joint Genome Institute.

Cotter, P. D., Gahan, C. G. and Hill, C. (2001). "A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid." Mol Microbiol 40(2): 465-75.

Cotter, P. D. and Hill, C. (2003). "Surviving the acid test: responses of gram-positive bacteria to low pH." Microbiol Mol Biol Rev 67(3): 429-53.

De Angelis, M. and Gobbetti, M. (2004). "Environmental stress responses in *Lactobacillus*: a review." Proteomics 4(1): 106-22.

De Angelis, M., Mariotti, L., Rossi, J., Servili, M., Fox, P. F., Rollan, G. and Gobbetti, M. (2002). "Arginine catabolism by sourdough lactic acid bacteria: purification and characterization of the arginine deiminase pathway enzymes from *Lactobacillus sanfranciscensis* CB1." Appl Environ Microbiol 68(12): 6193-201.

Diez-Gonzalez, F., Callaway, T. R., Kizoulis, M. G. and Russell, J. B. (1998). "Grain feeding and the dissemination of acid-resistant *Escherichia coli* from cattle." Science 281(5383): 1666-8.

Diez-Gonzalez, F. and Russell, J. B. (1999). "Factors affecting the extreme acid resistance of *Escherichia coli* O157:H7." Food Microbiol 16(4): 367-374.

Feehily, C. and Karatzas, K. A. (2013). "Role of glutamate metabolism in bacterial responses towards acid and other stresses." J Appl Microbiol 114(1): 11-24.

Foster, J. W. (2004). "*Escherichia coli* acid resistance: tales of an amateur acidophile." Nat Rev Microbiol 2(11): 898-907.

Frese, S. A., Benson, A. K., Tannock, G. W., Loach, D. M., Kim, J., Zhang, M., Oh, P. L., Heng, N. C., Patil, P. B., Juge, N., Mackenzie, D. A., Pearson, B. M., Lapidus, A., Dalin, E., Tice, H., Goltsman, E., Land, M., Hauser, L., Ivanova, N., Kyrpides, N. C. and Walter, J. (2011). "The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri.*" PLoS Genet 7(2): e1001314.

Galle, S. and Arendt, E. K. (2013). "Exopolysaccharides from sourdough lactic acid bacteria." Crit Rev Food Sci (inpress).

Gänzle, M. G., Ehmann, M. and Hammes, W. P. (1998). "Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of sourdough fermentation." Appl Environ Microbiol 64(7): 2616-23.

Gänzle, M. G. and Vogel, R. F. (2003). "Studies on the mode of action of reutericyclin." Appl Environ Microbiol 69(2): 1305-7.

Gärtner, K. (2002). "The forestomach of rats and mice, an effective device supporting digestive metabolism in muridae (review)." J Exp Anim Sci 42(1): 1-20.

Hersh, B. M., Farooq, F. T., Barstad, D. N., Blankenhorn, D. L. and Slonczewski, J. L. (1996). "A glutamate-dependent acid resistance gene in *Escherichia coli*." J Bacteriol 178(13): 3978-81.

Kaditzky, S. B., Behr, J., Stocker, A., Kaden, P., Gänzle, M. G. and Vogel, R. F. (2008). "Influence of pH on the formation of glucan by *Lactobacillus reuteri* TMW 1.106 exerting a protective function against extreme pH values." Food Biotechnol 22(4): 398-418.

Karatzas, K. A., Brennan, O., Heavin, S., Morrissey, J. and O'Byrne, C. P. (2010). "Intracellular accumulation of high levels of gamma-aminobutyrate by *Listeria monocytogenes* 10403S in response to low pH: uncoupling of gamma-aminobutyrate synthesis from efflux in a chemically defined medium." Appl Environ Microbiol 76(11): 3529-37.

Konings, W. N. (2002). "The cell membrane and the struggle for life of lactic acid bacteria." Anton Van Leeuw J Microb 82(1-4): 3-27.

Konings, W. N. and Otto, R. (1983). "Energy transduction and solute transport in streptococci." Anton Van Leeuw J Microb 49(3): 247-57.

Lin, J., Smith, M. P., Chapin, K. C., Baik, H. S., Bennett, G. N. and Foster, J. W. (1996). "Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*." Appl Environ Microbiol 62(9): 3094-100.

Lu, P., Ma, D., Chen, Y., Guo, Y., Chen, G. Q., Deng, H. and Shi, Y. (2013). "L-glutamine provides acid resistance for *Escherichia coli* through enzymatic release of ammonia." Cell Res 23(5): 635-44.

Ma, D., Lu, P. and Shi, Y. (2013). "Substrate selectivity of the acid-activated glutamate/gamma-aminobutyric acid (GABA) antiporter GadC from *Escherichia coli*." J Biol Chem 288(21): 15148-53.

Mazzoli, R., Pessione, E., Dufour, M., Laroute, V., Giuffrida, M. G., Giunta, C., Cocaign-Bousquet, M. and Loubiere, P. (2010). "Glutamate-induced metabolic changes in *Lactococcus lactis* NCDO 2118 during GABA production: combined transcriptomic and proteomic analysis." Amino Acids 39(3): 727-37.

Meroth, C. B., Walter, J., Hertel, C., Brandt, M. J. and Hammes, W. P. (2003). "Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis." Appl Environ Microbiol 69(1): 475-82.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res 29(9): e45.

Richard, H. and Foster, J. W. (2004). "*Escherichia coli* glutamate- and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential." J Bacteriol 186(18): 6032-41.

Rollan, G., Lorca, G. L. and Font de Valdez, G. (2003). "Arginine catabolism and acid tolerance response in *Lactobacillus reuteri* isolated from sourdough." Food Microbiol 20(3): 313-319.

Ryan, S., Begley, M., Gahan, C. G. and Hill, C. (2009). "Molecular characterization of the arginine deiminase system in *Listeria monocytogenes*: regulation and role in acid tolerance." Environ Microbiol 11(2): 432-45.

Schurr, B., Behr, J. and Vogel, R. (2013). "Role of the GAD system in hop tolerance of *Lactobacillus brevis*." Eur Food Res Technol: 1-9.

Schwab, C. and Gänzle, M. G. (2006). "Effect of membrane lateral pressure on the expression of fructosyltransferases in *Lactobacillus reuteri*." Syst Appl Microbiol 29(2): 89-99.

Sedgwick, G. W., Fenton, T. W. and Thompson, J. R. (1991). "Effect of protein precipitating agents on the recovery of plasma free amino acids." Can J Anim Sci 71(3): 953-957.

Stolz, P., Böcker, G., Hammes, W. P. and Vogel, R. F. (1995). "Utilization of electron acceptors by lactobacilly isolated from sourdough." Z Lebensm Unters Forsch 201: 91-96.

Stromeck, A., Hu, Y., Chen, L. and Gänzle, M. G. (2011). "Proteolysis and bioconversion of cereal proteins to glutamate and gamma-aminobutyrate (GABA) in Rye malt sourdoughs." J Agric Food Chem 59(4): 1392-9.

Su, M. S., Schlicht, S. and Gänzle, M. G. (2011). "Contribution of glutamate decarboxylase in *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation." Microb Cell Fact 10 Suppl 1: S8.

Thiele, C., Gänzle, M. G. and Vogel, R. F. (2002). "Contribution of sourdough lactobacilli, yeast, and cereal enzymes to the generation of amino acids in dough relevant for bread flavor." Cereal Chem 79(1): 45-51.

Toro, M., Arzt, E., Cerbon, J., Alegria, G., Alva, R., Meas, Y. and Estrada, S. (1987). "Formation of ion-translocating oligomers by nigericin." J Membr Biol 95(1): 1-8.

Tsai, M. F., McCarthy, P. and Miller, C. (2013). "Substrate selectivity in glutamate-dependent acid resistance in enteric bacteria." Proc Natl Acad Sci U S A 110(15): 5898-902.

Vermeulen, N., Gänzle, M. G. and Vogel, R. F. (2007). "Glutamine deamidation by cereal-associated lactic acid bacteria." J Appl Microbiol 103(4): 1197-205.

Vogel, R. F., Knorr, R., Muller, M. R., Steudel, U., Gänzle, M. G. and Ehrmann, M. A. (1999). "Non-dairy lactic fermentations: the cereal world." Anton Van Leeuw J Microb 76(1-4): 403-11.

Walter, J. (2008). "Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research." Appl Environ Microbiol 74(16): 4985-96.

Walter, J., Britton, R. A. and Roos, S. (2011). "Host-microbial symbiosis in the vertebrate gastrointestinal tract and the *Lactobacillus reuteri* paradigm." Proc Natl Acad Sci U S A 108 Suppl 1: 4645-52.

Walter, J., Schwab, C., Loach, D. M., Gänzle, M. G. and Tannock, G. W. (2008). "Glucosyltransferase A (GtfA) and inulosucrase (Inu) of *Lactobacillus reuteri* TMW1.106 contribute to cell aggregation, in vitro biofilm formation, and colonization of the mouse gastrointestinal tract." Microbiology 154(Pt 1): 72-80.

Ward, F. W. and Coates, M. E. (1987). "Gastrointestinal pH measurement in rats: influence of the microbial flora, diet and fasting." Lab Anim 21(3): 216-22.

Weingand-Ziadé, A., Gerber-Décombaz, C. and Affolter, M. (2003). "Functional characterization of a salt- and thermotolerant glutaminase from *Lactobacillus rhamnosus*." Enzyme Microb Technol 32(7): 862-867.

Wesney, E. and Tannock, G. (1979). "Association of rat, pig, and fowl biotypes of lactobacilli with the stomach of gnotobiotic mice." Microbial Ecol 5(1): 35-42.

Wieser, H. (2007). "Chemistry of gluten proteins." Food Microbiol 24(2): 115-9.

Wilson, C. M., Leach, D., O'Toole, P. W. and Tannock, G. W. (2011). "Inactivation of the *Lactobacillus reuteri* 100-23 ureC gene affects in vitro acid tolerance, and impairs ecological fitness in vivo." Book of Abstracts, 10th Symposium on Lactic Acid Bacteria. Egmond aan Zee.

CHAPTER FIVE: **GENERAL DISCUSSION AND CONCLUSIONS**

CHAPTER 5

5.1. General discussion

The ecological conditions that prevail during food fermentations, e.g. variable pH, carbohydrate availability, and anaerobic atmosphere provide a selective advantage for lactic acid bacteria, which are the dominating microflora of the majority of fermented foods. The aim of this study was to improve our understanding into the effects of the carbohydrate metabolism regulation and amino acid-based acid resistance in *Lactobacillus reuteri* in such ecological conditions and food quality.

5.1.1. Sucrose metabolism

In concordance to Schwab *et al.* (2007), this work showed that *L. reuteri* LTH5448 is able to metabolize sucrose via two pathways: employing a fructansucrase (levansucrase, FtfA) that produce exopolysaccharides (EPS) as alternative to sucrose hydrolysis; or the intracellular sucrose phosphorylase (ScrP) to gain energy. The deletion of the sucrose-inducible *scrP* had no impact on growth of *L. reuteri* LTH5448 in sucrose-containing MRS (sucMRS), but promoted a higher yield of levan, indicating that the lack of sucrose phosphorylase is compensated by an increased metabolic flux through levansucrase as sole sucrose-converting enzyme. Moreover, it was observed that both these enzymes play a role in the metabolism of raffinose family oligosaccharides (RFO), which are abundant carbohydrates in cereal and legumes giving *L. reuteri* LTH5448 an important competitive advantage.

Further studies on the regulation of sucrose phosphorylase and levansucrase demonstrated that these enzymes are induced by sucrose or raffinose, but not repressed by glucose. Regulation is mediated by sucrose regulator (ScrR); the deletion of *scrR* led to constitutive expression of sucrose phosphorylase and levansucrase, demonstrating that *scrR* is a substrate-controlled negative regulator. The lack of carbon catabolite repression of sucrose metabolic enzymes in *L. reuteri* differentiates this organism from other lactobacilli and likely reflects adaptation to cereal substrates where sucrose and raffinose are the major carbon sources in the rest grain.

5.1.2. Applications for improved food quality

EPS formed during cereal fermentation improves dough rheology and bread quality, acting as hydrocolloid replacers (Galle and Arendt, 2013). In gluten-free bread, hydrocolloids are added to mimic the properties of gluten. *In situ* synthesis of EPS by *L. reuteri* is a promising tool for the production of gluten-free products with improved quality characteristics and at the same time reducing the need for additives. For instance, Galle *et al.* (2012) showed that the formation of EPS levan by *L. reuteri* in sourdough improved bread volume, and delayed staling. Therefore, the use of an EPS- producing ScrP-negative *L. reuteri* strains may be beneficial in applications aiming a high yield of levan.

Another concern during production of gluten-free bread is that it typically results in products with low contents of micronutrients and dietary fiber. This may be compensated with the introduction of legume flours to a gluten-free formula

(Gallagher *et al.*, 2004). However, legume flours also contain a high level of RFO, which may be considered as an antinutritive factor. Therefore, a characterization of the metabolism of pulse flour oligosaccharides by *Lactobacillus* species in sourdough fermentations was performed. Levansucrase positive *L. reuteri* LTH5448 rapidly reduced the load of RFO with intermediate accumulation of α-galactooligosaccharides (α-GOS), which are hypothesized to modulate the composition and activity of intestinal microbiota. The conversion of these non-digestible oligosaccharides from antinutritive factors to functional ingredients by levansucrase leads to a gluten-free product with improved nutritional quality.

5.1.3. Amino acid-based acid resistance

The different ecological niches of *L. reuteri* require different ecological strategies, which also relate to acid resistance (Frese *et al.*, 2011). Aside from EPS formation, arginine deiminase (ADI) and glutamate decarboxylation (GAD) systems are involved in acid tolerance of *L. reuteri* (Dal Bello *et al.*, 2005; Kaditzky *et al.*, 2008; Su *et al.*, 2011). Glutamate activity in acid resistance of *L. reuteri* is dependent on glutaminases to convert glutamine to glutamate (Su *et al.*, 2011). Given the abundance of glutamine in wheat protein, this amino acid is likely to play a key role for the bacterial survival in sourdough fermentation. Further experiments demonstrated that the glutamine deamidation improves the acid tolerance of *L. reuteri* in pH-limited substrates (Zhang, 2011). Several lines of evidence suggest that these amino acid-based acid resistance mechanisms are

partially redundant in *L reuteri*; with respect to their function at different conditions; however, they showed to be complementary systems. It has been considered that arginine, glutamine or glutamate conversions consume intracellular protons and thus contribute to pH homeostasis and increase acid resistance (Cotter and Hill, 2003; Su *et al.*, 2011; Lu *et al.*, 2013). To elucidate the mechanisms underlying the effect of amino acid supplementation, the intracellular pH (pH_{in}) and transmembrane potential ($\Delta\Psi$) of *L. reuteri* cells were estimated. Remarkably, *L. reuteri* did not maintain a high intracellular pH under acidic conditions. The increase of the cytoplasmic pH was absent in presence of glutamine, and only a small variation was observed in presence of glutamate when compared to arginine, indicating that the membrane polarization may be a more relevant strategy for survival of *L. reuteri*.

These studies together provide novel insights into *L. reuteri*'s acid resistance mechanism, which involves glutamine deamidation followed by glutamate decarboxylation and allows competitiveness in acidic niches. Additionally, glutamine conversion by *L. reuteri* allows for the accumulation of the alternative end products glutamate, a taste-active compound, or the bioactive γ-aminobutyrate (GABA) (Stromeck *et al.*, 2011). The analysis of genetic determinants of glutamine conversion in lactobacilli thus facilitates the selection of starter cultures with a high potential to specifically affect not only the survival and general performance of bacterium in a food system, but also significantly improve its sensory quality.

5.2. Perspectives

L. reuteri constitute the natural microbiota of cereal fermentations, and its competitiveness has been attributed to the formation of organic acids and various structurally different antagonistic substances (Gänzle, 2004). This work focused on obtaining a better understanding of the factors contributing to the prevalence of specific *L. reuteri* strains in cereal fermentation; however, the results obtained can also explain the competitiveness of *L. reuteri* in intestinal habitats. Intestinal and sourdough *L. reuteri* strains are phylogenetically closely related, and equally well adapted for both the intestinal and sourdough milieu (Su *et al.*, 2012). Taken together, these observations offer new opportunities for the development of probiotic foods employing probiotic lactobacilli as starter cultures.

Specifically, this research provided insights into the impact of regulation of carbohydrate metabolism, and amino acid-based acid resistance in *L. reuteri* on strain competitiveness, contribution to food quality, and enhancement of functional compounds. In addition to the characteristics of *L. reuteri* for nutritional applications, results are also applicable to the use of *L. reuteri* as a probiotic, leading to further applications in functional food and feed (Table 5-1).

The ability to metabolize sucrose and RFO will contribute to *L. reuteri* ecological fitness in food fermentation, and also impacts the quality of fermented cereal products. The availability of carbohydrate in the upper intestine of grain-fed animals is remarkably similar to cereal fermentation, thus making those environments a similarly suitable substrate for *L. reuteri.* Sucrose metabolism contributes to the synthesis of EPS, which plays an important role in biofilm

formation and cell-cell adherence, and therefore has demonstrate to support EPS-producing strains, e.g. *L. reuteri*, to survive in intestinal passage and persist in the intestinal tract (Walter *et al.*, 2008; Sims *et al.*, 2011).

Table 5-1. Physiological traits of *Lactobacillus reuteri* that contribute to the competitiveness in sourdough or the gastrointestinal tract.

	Sourdough	**Intestine**
Sucrose metabolism	Increase competitiveness, production of EPS, improvement of sensory and structural properties, and acid resistance	Increase competitiveness, production of EPS, bacterial biofilm formation, and immunomodulation
Raffinose metabolism	Removal of RFO, and accumulation of α-GOS	Increase competitiveness
Amino acid metabolism	Survival long-term fermentation, and improvement of sensory quality	Survival gastric passage
Urea metabolism	None	Acid resistance
Glycerol utilization	Electron acceptor	Electron acceptor, biosynthesis of reuterin

Fructooligosaccharides (FOS) and galactooligosaccharides (GOS) are resistant to hydrolysis by human digestive enzymes but can be fermented by intestinal *Bifidobacterium* (Hammes and Dal Bello, 2002), which enhances the functionality of EPS as prebiotics. Similarly, the degradation of the RFO during *L. reuteri* fermentation helps alleviate the adverse effects of these complex carbohydrates in the digestibility of grain cereals, concomitantly with the generation of α-GOS, which are available as a substrate for intestinal microflora. The potential benefits of EPS and α-GOS formation by *L. reuteri* strains can be further evaluated for application as novel probiotic strains, isolated from cereal

fermented products, contributing to advancements in human nutrition, and consequently, health.

The ecological competitiveness of *L. reuteri* has been observed in continuous back-slopping sourdough fermentations and as a member of the commensal microbiota in humans and animals (Meroth *et al.*, 2003; Walter *et al.*, 2008). Both these habitats expose the organism to acidic stress, and the multitude of complementary or redundant acid resistance mechanisms implies that *L. reuteri* is well equipped to withstand acidic environments.

In addition to the contribution of EPS and amino acid systems to acid adaptation of *L. reuteri*, urea also plays a role on *L. reuteri*'s adaptation to the gastric niche. For instance, in some rodent isolates of *L. reuteri* urease activity contributes to acid resistance (Walter *et al.*, 2011; Wilson *et al.*, 2011). The competitiveness of *L. reuteri* has also been partly attributed to the formation of a low molecular weight broad-spectrum antimicrobial compound 3-hydroxypropionaldehydes, termed reuterin (Axelsson *et al.*, 1989). Reuterin is an intermediate metabolite of glycerol, which is further used as a hydrogen acceptor in co-fermentation with glucose, regenerating NAD^+ from NADH, and thus contributing to improved growth yield of *L. reuteri* (Talarico *et al.*, 1990; Luthi-Peng *et al.*, 2002). In *L. reuteri*, human isolates but not rodent isolates metabolize glycerol and produce reuterin as intermediate (Frese *et al.*, 2011; Walter *et al.*, 2011). Genetic differences between rodents and human-adapted strains of *L. reuteri* may reflect that residents of the rodent forestomach but not human colonic microbiota are exposed to a nutrient-rich environment, indicating that this

metabolic pathway contributes to the ecological adaptation to specific habitats (Walter *et al.*, 2011).

Finally, this study provided some insight on characteristics of *L. reuteri* that are beneficial in cereal fermentation. These *L. reuteri* strains demonstrate that fermented cereal products can be modified to produce food and feed with high cell counts of probiotic organisms. The potentially probiotic activities of some LAB represent an attractive field of research and a promising opportunity to promote advancement in the food industry. In the prospective future, cereal fermentation may be used to modify cereal substrates and generate nutritionally tailored functional foods and feed.

5.3. References

Axelsson, L. T., Chung, T. C., Dobrogosz, W. J. and Lindgren, S. E. (1989). "Production of a broad spectrum antimicrobial substance by *Lactobacillus reuteri*." Microb Ecol Health D 2(2): 131-136.

Cotter, P. D. and Hill, C. (2003). "Surviving the acid test: responses of gram-positive bacteria to low pH." Microbiol Mol Biol Rev 67(3): 429-53, table of contents.

Dal Bello, F., Walter, J., Roos, S., Jonsson, H. and Hertel, C. (2005). "Inducible gene expression in *Lactobacillus reuteri* LTH5531 during type II sourdough fermentation." Appl Environ Microbiol 71(10): 5873-8.

Frese, S. A., Benson, A. K., Tannock, G. W., Loach, D. M., Kim, J., Zhang, M., Oh, P. L., Heng, N. C., Patil, P. B., Juge, N., Mackenzie, D. A., Pearson, B. M., Lapidus, A., Dalin, E., Tice, H., Goltsman, E., Land, M., Hauser, L., Ivanova, N., Kyrpides, N. C. and Walter, J. (2011). "The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri.*" PLoS Genet 7(2): e1001314.

Gallagher, E., Gormley, T. and Arendt, E. (2004). "Recent advances in the formulation of gluten-free cereal-based products." Trends Food Sci Tech 15(3-4): 143-152.

Galle, S. and Arendt, E. K. (2013). "Exopolysaccharides from sourdough lactic acid bacteria." Crit Rev Food Sci Nutr (in press).

Galle, S., Schwab, C., Dal Bello, F., Coffey, A., Gänzle, M. G. and Arendt, E. K. (2012). "Influence of in-situ synthesized exopolysaccharides on the quality of gluten-free sorghum sourdough bread." Int J Food Microbiol 155(3): 105-12.

Gänzle, M. G. (2004). "Reutericyclin: biological activity, mode of action, and potential applications." Appl Microbiol Biotechnol 64(3): 326-32.

Hammes, W. P. and Dal Bello, F. (2002). "How selective are probiotics?" Senate Commission on Food Safety. F. F. S. Aspects. Weinheim, Germany, Wiley-VCH.

Kaditzky, S. B., Behr, J., Stocker, A., Kaden, P., Gänzle, M. G. and Vogel, R. F. (2008). "Influence of pH on the formation of glucan by *Lactobacillus reuteri* TMW 1.106 exerting a protective function against extreme pH values." Food Biotechnol 22(4): 398-418.

Lu, P., Ma, D., Chen, Y., Guo, Y., Chen, G. Q., Deng, H. and Shi, Y. (2013). "L-glutamine provides acid resistance for *Escherichia coli* through enzymatic release of ammonia." Cell Res 23(5): 635-44.

Luthi-Peng, Q., Dileme, F. B. and Puhan, Z. (2002). "Effect of glucose on glycerol bioconversion by *Lactobacillus reuteri.*" Appl Microbiol Biotechnol 59(2-3): 289-96.

Meroth, C. B., Walter, J., Hertel, C., Brandt, M. J. and Hammes, W. P. (2003). "Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis." Appl Environ Microbiol 69(1): 475-82.

Schwab, C., Walter, J., Tannock, G. W., Vogel, R. F. and Gänzle, M. G. (2007). "Sucrose utilization and impact of sucrose on glycosyltransferase expression in *Lactobacillus reuteri.*" Syst Appl Microbiol 30(6): 433-43.

Sims, I. M., Frese, S. A., Walter, J., Loach, D., Wilson, M., Appleyard, K., Eason, J., Livingston, M., Baird, M., Cook, G. and Tannock, G. W. (2011). "Structure and functions of exopolysaccharide produced by gut commensal *Lactobacillus reuteri* 100-23." ISME J 5(7): 1115-24.

Stromeck, A., Hu, Y., Chen, L. and Gänzle, M. G. (2011). "Proteolysis and bioconversion of cereal proteins to glutamate and gamma-aminobutyrate (GABA) in Rye malt sourdoughs." J Agric Food Chem 59(4): 1392-9.

Su, M., Schlicht, S. and Gänzle, M. (2011). "Contribution of glutamate decarboxylase in *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation." Microb Cell Fact 10(Suppl 1): S8.

Su, M. S., Oh, P. L., Walter, J. and Gänzle, M. G. (2012). "Intestinal origin of sourdough *Lactobacillus reuteri* isolates as revealed by phylogenetic, genetic, and physiological analysis." Appl Environ Microbiol 78(18): 6777-80.

Talarico, T. L., Axelsson, L. T., Novotny, J., Fiuzat, M. and Dobrogosz, W. J. (1990). "Utilization of glycerol as a hydrogen acceptor by *Lactobacillus reuteri*: purification of 1,3-propanediol:NAD oxidoreductase." Appl Environ Microbiol 56(4): 943-8.

Walter, J., Britton, R. A. and Roos, S. (2011). "Host-microbial symbiosis in the vertebrate gastrointestinal tract and the *Lactobacillus reuteri* paradigm." Proc Natl Acad Sci U S A 108 Suppl 1: 4645-52.

Walter, J., Schwab, C., Loach, D. M., Gänzle, M. G. and Tannock, G. W. (2008). "Glucosyltransferase A (GtfA) and inulosucrase (Inu) of *Lactobacillus*

reuteri TMW1.106 contribute to cell aggregation, in vitro biofilm formation, and colonization of the mouse gastrointestinal tract." Microbiology 154(Pt 1): 72-80.

Wilson, C. M., Leach, D., O'Toole, P. W. and Tannock, G. W. (2011). "Inactivation of the *Lactobacillus reuteri* 100-23 ureC gene affects in vitro acid tolerance, and impairs ecological fitness in vivo." Book of Abstracts, 10th Symposium on Lactic Acid Bacteria. Egmond aan Zee.

Zhang, C. (2011). "Metabolism of lactic acid bacteria in wheat sourdough and bread quality." Edmonton, Canada, University of Alberta. PhD.

APPENDIX: **BIOTECHNOLOGY APPLICATION**

This chapter includes experimental work conducted from 2010 to 2012 with results not suitable for publication. Continuation with a completely revised experimental design was abandoned in favour of experiments related to amino acid based acid resistance (Chapeter 4) as these were considered more relevant.

APPENDIX

A.1. Xylose metabolism

Xylose is one of the most abundant monosaccharide in nature, being the major backbone of plant hemicellulose, where it is found in the polysaccharide form, such as xylan (Chandel *et al.*, 2010**)**. The hydrogenation of xylose results in xylitol, a sugar alcohol that is used as a sugar substitute and building block for organic synthesis (Werpy and Perterson, 2004). Although some microorganisms naturally produce xylitol, for large scale commercial use it needs to be manufactured by a chemical hydrogenation, which is a nonselective process that requires high temperature and pressure, as well as pure xylose, which makes it an expensive process (Granstrom *et al.*, 2007).

Xylose metabolism by bacteria, fungi and yeasts through the oxido-reductive pathway forms xylitol as a metabolic intermediate. The main enzyme involved in this process is xylitol reductase (XR), a NAD(P)H-dependent enzyme. Under oxygen-limiting conditions, the NADH coenzyme cannot be completely re-oxidized to NAD by the respiratory chain, and the intracellular concentration of NADH favors xylitol accumulation (Granstrom *et al.*, 2007). Among the natural xylitol producers *Candida* spp. and *Pichia stipis* have been pointed out as model xylitol producers (Akinterinwa *et al.*, 2008).

Considering the industrial applications of xylitol, the development of alternative ways to achieve its production by food-grade microorganisms, such as lactic acid bacteria (LAB), would be an attractive choice in the food industry. Many attempts were undertaken to obtain xylitol production by non-xylitol producers, such as LAB. Nyyssola *et al.* (2005) constructed a *Lactococcus lactis*

mutant that overexpresses XR from *Pichia stipitis* and a xylose transporter from *Lactobacillus brevis*. In that work, overexpression of XR, concomitant with the expression of the xylose transporter, improved xylose influx and xylitol accumulation. However, the limited NADH availability suppressed the xylose conversion and consequently the growth of *Lactococcus lactis*. This limitation may be circumvented by using heterofermentative LAB as host for XR. These generate an abundant surplus of the cofactor NADH in the pentose-phosphate pathway (Figure A-1).

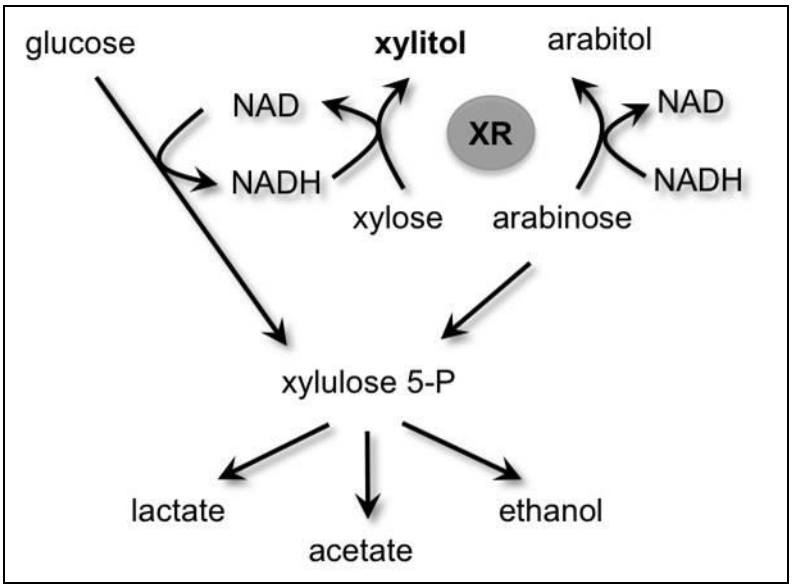


Figure A-1. Proposed pathway of heterofermentative LAB xylose reductase (XR) acting on xylose with NADH. Cofactor regeneration drives glucose metabolism in the pentose-phosphate pathway to achieve enhanced conversion of xylitol.

A.2. Development of an effective biocatalyst for the conversion of xylose to xylitol through metabolic engineering of LAB

A.2.1. Suitable host strains of LAB for the selective conversion of xylose into xylitol

In order to select a suitable host organism, about 50 wild-type strains obtained from the food microbiology laboratory strain collection at the University of Alberta (Edmonton, Canada) (Table A-1) were screened with respect to xylose and arabinose utilization. Pentose metabolism was determined in modified deMan-Rogosa-Sharp medium (mMRS) (Stolz *et al.*, 1995) in the presence of 20 g/L glucose (gluMRS), or 20 g/L arabinose and glucose 2 g/L (arabMRS) or 20 g/L xylose and glucose 2 g/L (xylMRS) as carbon sources (Table A-2). The kinetics of substrate consumption and product formation from glucose and pentoses was determined by HPLC. Four strains were selected based on the utilization of arabinose but not xylose, xylose but not arabinose, or both: *Lactobacillus brevis* FUA 3039, *Lactobacillus brevis* FUA 3113, *Lactobacillus brevis* FUA 3161, respectively. *Lactococcus lactis* MG1363 was used as control.

APPENDIX

Table A-1. Strains used for characterization with respect to pentose transport and utilization

FUA #	Species	Sources
3007	*Lactobacillus hammesii*	Sourdough
3008	*Lactobacillus nantensis*	Sourdough
3016*	*Lactococcus lactis*	Fermented milk product
3025	*Lactobacillus reuteri* LTH2584	Sourdough
3026	*Lactobacillus reuteri* TMW1.106	Sourdough
3027	*Lactobacillus reuteri* TMW1.112	Sourdough
3028	*Lactobacillus reuteri* TMW1.1656	Sourdough
3029	*Lactobacillus reuteri* LTH5448	Sourdough
3030	*Lactobacillus reuteri* 100-23	Rodent intestine
3031	*Lactobacillus reuteri* TMW1.1268	Rodent intestine
3033	*Lactobacillus reuteri* LTH5794	Human intestine
3034	*Lactobacillus reuteri* LTH5795	Human intestine
3035	*Lactobacillus reuteri* LTH5796	Human intestine
3036	*Lactobacillus reuteri* LTH5797	Human intestine
3037	*Lactobacillus reuteri* LTH5798	Human intestine
3039*	*Lactobacillus brevis*	Sourdough (MGG)
3042	*Lactobacillus reuteri*	Mouse intestine
3043	*Lactobacillus reuteri*	Mouse intestine
3044	*Lactobacillus reuteri*	Mouse intestine
3048	*Lactobacillus reuteri*	Mouse intestine
3049	*Lactobacillus vaginalis*	Mouse intestine
3060	*Leuconostoc gelidum*	Sausage
3090	*Leuconostoc mesenteroides*	Kvass
3092	*Lactococcus lactis*	Bear feces
3097	*Leuconostoc mesenteroides*	Meat
3101	*Leuconostoc mesenteroides*	Buffalo meat
3105	*Weissella confusa* ATCC10881	n.d.
3106	*Weissella minor*	n.d.
3113*	*Lactobacillus brevis*	Sourdough, Alpine bakery Whitehorse
3120	*Weissella cibaria*	Ropy (dextransucrase)
3121	*Lactobacillus paralimentarius*	Sourdough, Salt spring Island Bakery
3124	*Lactobacillus rossiae*	Sourdough, Salt spring Island Bakery
3125	*Lactobacillus spicheri*	Sourdough, Salt spring Island Bakery
3126	*Lactobacillus rossiae*	n.d.
3143	*Leuconostoc mesenteroides*	Cow vagina
3148	*Lactobacillus reuteri* ATCC2372	n.d.
3161*	*Lactobacillus brevis*	Sourdough household 2.3
3162	*Lactobacillus rossiae*	Sourdough household 4.4
3165	*Lactobacillus fermentum*	Ting
3169	*Lactobacillus parabuchei*	Ting
3170	*Lactobacillus buchneri*	Ting
3177	*Lactobacillus fermentum*	Whey
3182	*Leuconostoc mesenteroides*	vilii
3184	*Leuconostoc mesenteroides*	vilii
3188	*Leuconostoc mesenteroides*	vilii
3189	*Leuconostoc mesenteroides subsp. cremois*	n.d.
3193	*Leuconostoc mesenteroides*	n.d.
3199	*Lactobacillus harbinensis*	n.d.
3218	*Leuconostoc mesenteroides subsp. cremois*	kimchii
3219	*Leuconostoc mesenteroides subsp. cremois*	kimchii

Note: n.d., not determined; *****: selected strains

APPENDIX

Table A-2. Screening of 50 wild-type strains. Shown is the number of strains in the species exhibiting the respective fermentation pattern

Species	+ + +	+ + –	+ – +	+ – –
Lactobacillus brevis	1	1		1
Lactobacillus buchneri	1			
Lactobacillus fermentum		2		
Lactobacillus hammesii	1			
Lactobacillus harbinensis		1		
Lactobacillus nantensis				1
Lactobacillus parabuchei		1		
Lactobacillus paralimentarius				1
Lactobacillus reuteri	1	7	2	7
Lactobacillus rossiae				3
Lactobacillus spicheri	1			
Lactobacillus vaginalis	1			
Lactococcus lactis	1	1		
Leuconostoc gelidum		1		
Leuconostoc mesenteroides	1	2		8
Weissella cibaria	1			
Weissella confusa			1	
Weissella minor				1

Note: + + +, growth in MRS, arabMRS and xylMRS; + + –, growth in MRS and arabMRS; + – + growth in MRS and xylMRS; + – –, growth in MRS.

A.2.2. Construction of a LAB enable to convert xylose to xylitol

The *Escherichia coli – Lactococcus lactis* expression shuttle vector pAMJ586 (Bioneer A/S, Hørsholm, Denmark) was used for cloning of the XR gene (*xylR*). Plasmids harboring XR genes (*xylR*) from *C. tenuis* CBS 4435 and *P. stipitis* CBS 5773 (Centraalbureau voor Schimmelcultures, Netherlands) – obtained from a previous work (Zhan *et al.*, 2006) – were used as PCR templates for amplification using primers listed in Table A3.

Restriction digest of the PCR products was then conducted to generate the insert with FastDigest® restriction enzymes SmaI and SalI (Fermentas, Burlington, Canada). The same restriction digest was done for the pAMJ586 shuttle vector and all SmaI and SalI restriction fragments were gel purified using the QIAquick Gel Extraction Kit (Qiagen) after electrophoresing in a 1% agarose gel at 90 V for 45 minutes. Ligation using the T4 DNA ligase (Fermentas) was done to produce the final construct, which was named as pCtXR-RBS1 or pCtXR-RBS4. The recombinant plasmid was transformed into *E. coli* TG1 (Stratagene, Amsterdam, the Netherlands) via electroporation to first increase the yield of pCtXR, and then it was subsequently transformed into the XR negative wild-type LAB strains previously selected. The electroporation conditions used were 25 μF, 2 kV, and 200 Ω in 0.1 cm Gene Pulser® cuvetter (Biorad, Mississauga, Canada). Electroporated *E. coli* cells were recovered in SOC medium (Life technologies) and electroporated *Lactococcus lactis* and *Lactobacillus brevis* cells were recovered in M17 and mMRS, respectively. After recovery, transformed strains were grown on their respective media with

erythromycin as the selective agent at (200 mg/L in LB) for *E. coli* and (10 mg/L in M17 and mMRS) for LAB strains. Successful cloning of the *xylR* gene was confirmed by PCR (primers Ct-F and Ct-R, Table A3) and sequencing by Macrogen (Macrogen, Rockville, USA).

Table A-3. Oligonucleotide primers used in xylose reductase amplification

Primer name	5' – 3'	Application
Ct-F - RBS1	TCC*CCCGGG*TCTAGATTAGGGTAACTTTGAAAGGATATTCCTC **ATG**AGCGCAAGTATCC	Insert primer
Ct-F - RBS4	TCC*CCCGGG*AAATTACATATTTTATTTTGGAGGAAGAAAGATT **ATG**AGCGCAAGTATCC	Insert primer
Ct-R - SalI	ACGC*GTCGAC*AGATTGGAATGTTGTCC	Insert primer
Ct-F	**ATG**AGCGCAAGTATCC	Sequencing
Ct-R	AGATTGGAATGTTGTCC	Sequencing

Note: Italic: restriction site; underlined: ribosome binding site (RBS); bold: start codon.

Table A-4. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics
	Strains
E. coli TG1	Cloning host for pAMJ586-derivative plasmids
Lactococcus lactis	FUA 3016, fermented milk product isolate
Lactobacillus brevis	FUA 3039, sourdough isolate
Lactobacillus brevis	FUA 3113, sourdough isolate
Lactobacillus brevis	FUA 3161, sourdough isolate
Lactococcus lactis 3016CtXR	Wild type strain containing pAMJ586CtXR
Lactobacillus brevis 3039CtXR	Wild type strain containing pAMJ586CtXR
Lactobacillus brevis 3161CtXR	Wild type strain containing pAMJ586CtXR
	Plasmids
pAMJ586	Shuttle vector used in the hosts *E. coli* and LAB, Erm^r
pCtXR-RBS1	pAMJ586 containing RBS1-Ct*xylR*, Erm^r
pCtXR-RBS4	pAMJ586 containing RBS4-Ct*xylR*, Erm^r

Note: Erm^r, erythromycin resistance

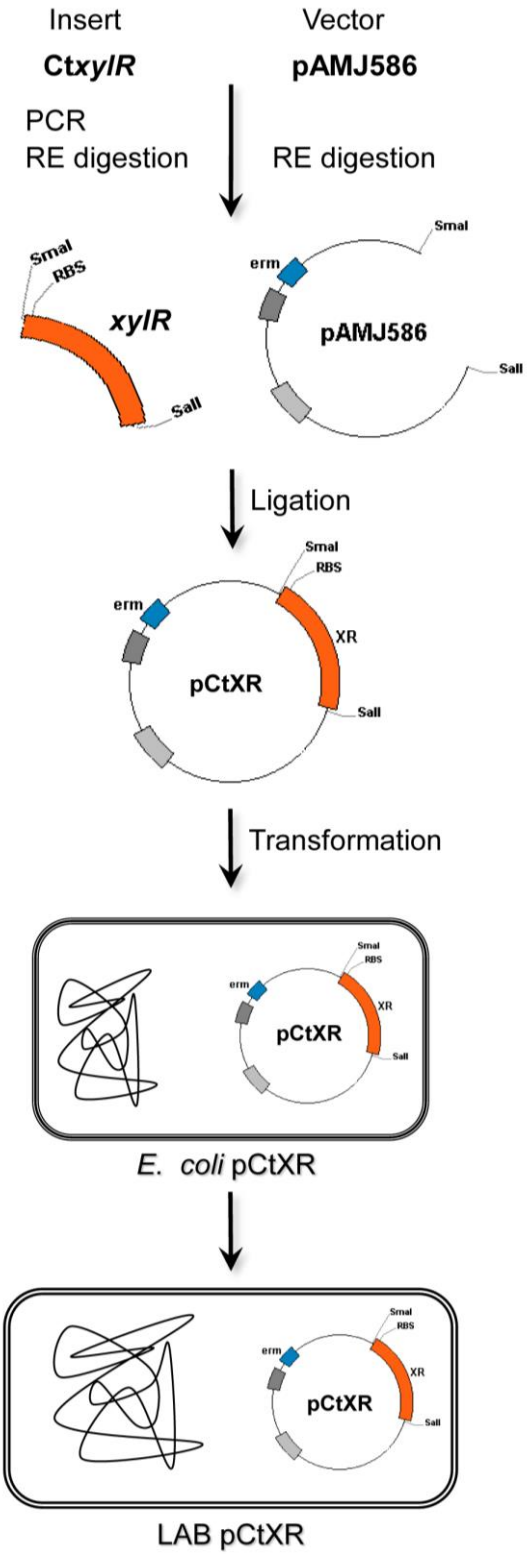


Figure A-2. Genetic engineering of a LAB enables conversion of xylose in xylitol.

A.2.3. Characterization of xylitol producer

Unless otherwise indicated, the LAB recombinant strains were anaerobically cultivated at 30°C in mMRS in presence of erythromycin (5 mg/L in liquid medium, 10 mg/L in agar plates). For preparation of working cultures, strains were obtained from -80°C stock cultures, streaked on mMRS plates, and subcultured in mMRS prior to use.

A.2.3.1.Determination of XR activity in crude cell extract (CCE)

Cells grown overnight were harvested by centrifugation (5000 × *g*, 10 min, 4 °C). The cells resuspension were washed once in sodium phosphate buffer (PB) (50 mM, pH 6.5), resuspended in PB with 10% glycerol and 1 mM $MgCl_2$, and disrupted using a bead beater at 4 °C. Crude cell extracts (CCE) were collected after centrifuging (12000 × *g*, 10 min, 4 °C). Enzymatic assays were conducted according to Kern *et al.* (1997). Measurements were performed every 30 seconds until 5 minutes was reached. Absorption at 340 nm was determined using a Varioskan Flash Multimode Reader (Fisher Scientific Limited, Ottawa, Canada). CCE of LAB without plasmid was included in all assays. None of the wild-type and recombinants strains listed in Table A-4 showed xylose reductase activity.

A.2.3.2.Organic acid analysis in buffer fermentation

Cells grown overnight were washed once in potassium phosphate buffer (50 mM, pH 6.5), and then resuspended in the same buffer containing xylose (50 mM) or fructose (50 mM) in addition to glucose (25 mM). Buffer fermentation

was assessed at 30 °C, and samples were taken for metabolic analysis after 3 and 6 hours of incubation. Samples were centrifuged at 5000 × *g* for 10 min to collect the supernatants, which were then precipitated by being mixed with an equal volume of 7% perchloric acid at 4°C overnight. Precipitates were removed by centrifugation (10000 × *g*, 10 min, 4°C). Organic acids and ethanol were identified by high-performance liquid chromatography (HPLC). None of the recombinants strains listed in Table A-4 produced xylitol. The increased accumulation of acetate – that would indicate the reduction of xylose to xylitol for regeneration of reduced co-factors - was also absent. Furthermore, *L. brevis* FUA3161 strain performed homolactic fermentation, and was further identified by sequencing of 16S rRNA as *Lactobacillus plantarum.*

A.3. Future work

Although xylose reductase from the yeasts *Pichia stipitis* and *Candida tenuis* are considered a model xylitol enzyme producer, these enzymes have higher catalytic efficiencies toward L-arabinose than D-xylose. In the future work, a more substrate-specific enzyme could be employed. The XR enzyme of *Neurospora crassa* (NxXR) had a higher selectivity for D-xylose over L-arabinose, compared with other XRs (Woodyer *et al.*, 2005). To improve the selectivity toward xylitol production, Nair and Zhao (2008, 2010) engineered the NcXR for reduced L-arabinose reductase activity and characterized a mutant enzyme NcXR that had a 50-fold lower catalytic efficiency to L-arabinose. An engineered *E. coli* strain expressing this mutated XR produced xylitol but not

arabinitol from an equiweight mixture of xylose, arabinose, and glucose. Therefore, the isolated NcXR might be a better choice for engineering due to its higher preference for xylose. Gene replacement of mannitol dehydrogenase by the highly selective XR from *Neurospora crassa* using the double crossover method employed on the Chapter 3 of this thesis could be an alternative approach to provide an efficient route toward the production of pure xylitol starting with a mixed carbon source. Replacement of mannitol dehydrogenase in thermophilic and obligate heterofermentative lactobacilli would allow the constitutive expression, and would eliminate the ability of the host strains to produce mannitol.

A.4. References

Akinterinwa, O., Khankal, R. and Cirino, P. C. (2008). "Metabolic engineering for bioproduction of sugar alcohols." Curr Opin Biotechnol 19(5): 461-7.

Chandel, A., Singh, O. and Venkateswar Rao, L. (2010). "Biotechnological applications of hemicellulosic derived sugars: state-of-the-art." Sustainable Biotechnology. O. V. Singh and S. P. Harvey, Springer Netherlands: 63-81.

Granstrom, T. B., Izumori, K. and Leisola, M. (2007). "A rare sugar xylitol. Part I: the biochemistry and biosynthesis of xylitol." Appl Microbiol Biotechnol 74(2): 277-81.

Kern, M., Haltrich, D., Nidetzky, B. and Kulbe, K. D. (1997). "Induction of aldose reductase and xylitol dehydrogenase activities in *Candida tenuis* CBS 4435." FEMS Microbiol Lett 149(1): 31-7.

Nair, N. U. and Zhao, H. (2008). "Evolution in reverse: engineering a D-xylose-specific xylose reductase." ChemBioChem 9(8): 1213-5.

Nair, N. U. and Zhao, H. (2010). "Selective reduction of xylose to xylitol from a mixture of hemicellulosic sugars." Metab Eng 12(5): 462-8.

Nyyssola, A., Pihlajaniemi, A., Palva, A., von Weymarn, N. and Leisola, M. (2005). "Production of xylitol from D-xylose by recombinant *Lactococcus lactis*." J Biotechnol 118(1): 55-66.

Stolz, P., Böcker, G., Hammes, W. and Vogel, R. (1995). "Utilization of electron acceptors by lactobacilli isolated from sourdough." Z Lebensm Unters Forsch 201(1): 91-96.

Werpy, T. and Perterson, G. (2004). "Top value added chemicals from biomass, volume I: results of screening for potential candidates from sugars and synthesis gas." Retrieved June 11, 2013, 2013, from http://www1.eere.energy.gov/bioenergy/pdfs/35523.pdf.

Woodyer, R., Simurdiak, M., van der Donk, W. A. and Zhao, H. (2005). "Heterologous expression, purification, and characterization of a highly active xylose reductase from *Neurospora crassa*." Appl Environ Microbiol 71(3): 1642-7.

Zhan, K., Bressler, D. C. and Gänzle, M. G. (2006). "Heterologous and heterofermentative expression of xylose reductase in *Escherichia coli* and *Lactobacillus reuteri*." Unpublished data.