University of Alberta

Metabolism of Lactic Acid Bacteria in Wheat Sourdough and Bread Quality

by

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Dedication

I would like to dedicate this thesis to my mother and my wife. Thanks for always supporting me during the hard time. Without you I couldn't have gone so far.

Abstract

Study on metabolism of lactic acid bacteria in wheat sourdough can improve bread quality such as bread flavour, texture and shelf life. This dissertation focused on several metabolic pathways in *Lactobacillus* strains on both biochemical and genetic level to fulfill three objectives.

The first objective was to produce bread preservative propionate from lactate by cometabolism of *Lactobacillus buchneri* and *Lactobacillus diolivorans* to extend bread shelf life. The results showed that propionate was formed in cofermentation. Bread containing 20% sourdough from coferementation effectively inhibited growth of three of the four selected moulds for more than 12 days compared to traditional sourdough. The use of 10% experimental sourdough deferred growth of two molds by one day.

The second objective was to characterize the metabolism of α-ketoglutarate (α-KG) in sourdough strains. Alpha-KG is an important amino group acceptor for flavour production during sourdough fermentation, however, knowledge on metabolism of α-KG in sourdough strains is lacking. Study of metabolism of α-KG in *Lactobacillus sanfranciscensis* and *Lactobacillus reuteri* showed that *L. sanfranciscensis* and *L. reuteri* utilized α-KG as electron acceptor, which was converted to 2-hydroxyglutarate in both media and wheat sourdough. The presence of phenylalanine and citrate in addition to α-KG partially redirected the use of α-KG from electron acceptor to amino group acceptor.

The third objective was to compare the acid resistance pathways in *L. reuteri* 100-23. Inducible gene expressions for acid resistance are important survival strategy for *Lactobacillus*. Genes encoding three glutaminases in *L. reuteri* 100-23 were studied and compared with *adi* and *gadB* to find out the responsible acid resistance pathway under different acidic conditions. Under pH 3.5, ADI pathway is effective in acid resistance. Expression of *gls3* was the highest during acid resistance among the three glutaminase genes. Analysis of gene expression in Δ*gadB* and Δ *gls*3 strains showed that deletion of *gadB* resulted in over-expression of *adi* and *gls3*, whereas deletion of *gls3* showed over-expression of *gls1*, *gls2* and *gadB* compared to wild type strains. Gene expression in wheat sourdough showed comparable over-expression of *gls3* and *gadB*.

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Abbreviations

α-KG:	α- ketoglutarate
ADI:	arginine deiminase
ALS:	acetolactate synthase
CK:	carbamate kinase
C-S lyase:	cystathionine lyase
EPS:	exopolysacchrides
FA:	fatty acid
GABA:	γ-aminobutyrate
GAD:	glutamate decarboxylase
GC:	gas chromatography
GI:	gastrointestinal tract
HePS:	heteropolysacchrides
HoPS:	homopolysacchrides
HPLC:	High performance liquid chromatography
LAB:	lactic acid bacteria
MIC:	minimum inhibitory concentration
mMRS:	modified MRS
2-OHG:	2-hydroxyglutarate
OPA:	o-phthaldialdehyde
OTC:	ornithine transcarbamoylase
RB:	reaction buffer
r. m.	relative moisture
RT-qPCR:	reverse transcription quantative PCR

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Chapter 1: Introduction and literature review

1.1. Use of sourdough in bread production.

Bread delivers to consumers an attractive, palatable and digestible form of cereals (Chamberlain, 1975). Two methods of dough making have been traditionally practiced: the straight dough process and sourdough. Processing of straight dough involves the leavening of dough by yeast only, whereas sourdough is fermented by a combination of yeast and lactic acid bacteria (LAB), typically *Lactobacillus* spp., for leavening and acidification (Figure 1.1). Sourdough preparation is one of the oldest food fermentation technologies in the world, with several thousand years of history dating back to ancient Egypt. Compared to bread leavened only by baker's yeast, combined use of LAB and yeast improves bread qualities such as flavour, texture, and shelf life (Figure 1.2) (Moroni et al, 2009; Arendt et al, 2007). During traditional sourdough fermentation, LAB typically reach a concentration of 10^9 cfu g^{-1} of dough with a ratio of 100:1 to yeast (Hammes et al, 2005; Gobbetti, 1998). Modern sourdough fermentation more often starts with an initial fermentation using lactobacilli in dough to provide desired dough quality, followed by leavening of dough by yeast. Based on the technology of dough processing applied, sourdough can be divided into four types: Type 0, I, II, and III (De Vuyst and Neysens, 2005) (Figure 1.1). Type 0 sourdough is sponge dough started by baker's yeast. Type I sourdough is used primarily for leavening, and is maintained by continuous propagation, using lower temperatures and shorter fermentation times. Type II sourdough is typically used as a baking improver, with use of higher temperatures and longer times for more efficient fermentation compared to type I sourdough. Different LAB strains dominate type I and II

sourdough, and wheat sourdough fermented by those LAB results in a pH range from 3.5-4.3 (Clarke and Arendt, 2005; Collar et al, 1994; Wehrle and Arendt, 1998; Thiele et al, 2002). *Lactobacillus sanfranciscensis* is the dominant species isolated from Type I sourdough, and *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus paralimentarius*, and *Lactobacillus rossiae* are also commonly isolated. *Lactobacillus amylovorus*, *Lactobacillus fermentum*, *Lactobacillus pontis*, and *Lactobacillus reuteri* are common isolates from Type II sourdough (De Vuystet et al, 2009). Dried starter culture is commonly referred as Type III sourdough (Hammes and Gänzle, 1998; Böcker et al, 1995). Some famous sourdough products commonly consumed include Colomba, San Francisco sourdough bread, and French baguette (Arendt et al, 2007).

Sourdough plays an important role in rye bread. It is an essential tool to produce bread with enhanced volume, since acidification from sourdough fermentation inhibits hydrolysis of starch by amylase and solubilizes rye pentosan (De Vuyst and Vancanneyt, 2007). Bread volume of wheat sourdough bread is maintained effectively by the gluten network (Brandt 2007). However, use of sourdough fermentation in wheat bread improves flavour, texture, and shelf life as previously reviewed by Clarke and Arendt (2005). Rye bread is consumed mainly in central and northern Europe but consumption of wheat bread is world-wide. Wheat is the third most-harvested cereal in the world and is thus worthy of intensive study (ProdSTAT. FAOSTAT. Retrieved on 2011-02-14).

Besides the positive effects of sourdough fermentation by LAB on bread texture, flavour, and shelf life, sourdough fermentation by LAB also contributes to the

nutritional value of bread, including production of prebiotic dextran and fiber, starch with low glycemic index, vitamin enhancement, and phytate reduction, as reviewed by Moroni et al, (2009); Poutanen et al, (2009); Katina et al, (2005). Additionally, one of the end products from glutamine metabolism, γ-aminobutyrate (GABA), possesses antihypertensive activity (Rizzello et al, 2008a). Several studies have been conducted in order to produce GABA as functional product (Komatsuzaki et al, 2005, 2008; Siragusa et al, 2007; Rizzello et al, 2008a, b). Sourdough fermented with *L. reuteri* produced around 90 mM kg^{-1} DM GABA (Stromeck et al, 2011).

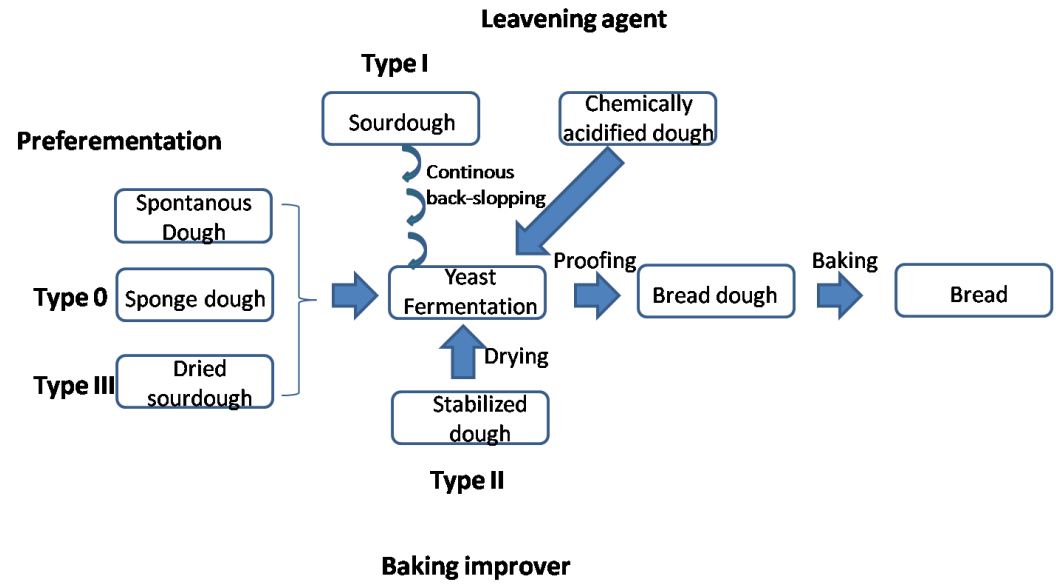

Figure 1.1. Typical process for production of sourdough bread.

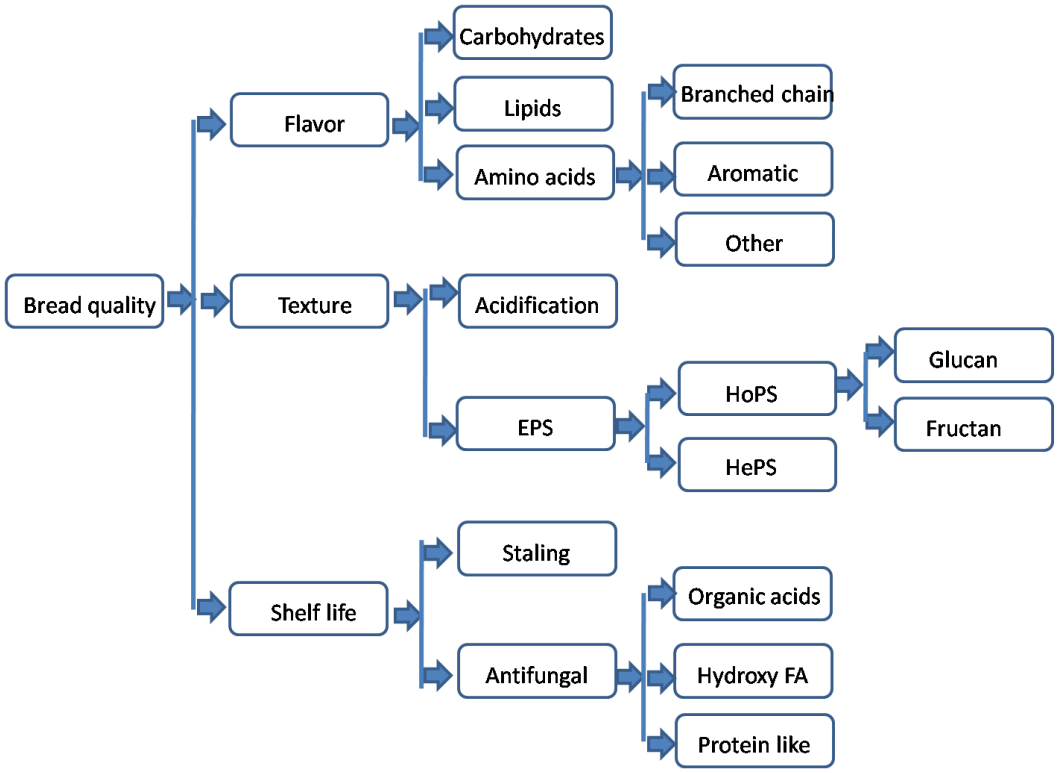

Figure 1. 2. Factors that affect bread quality. EPS: exopolysacchride; HoPS: homopolysacchrides; HePS: heteropolysacchrides; FA: fatty acid

1.2. LAB metabolism in sourdough and bread flavour.

1.2.1. Introduction of sourdough bread flavour.

Fermentation of dough greatly enhances flavour compounds (Frasse et al, 1993). Flavour of sourdough bread refers to both aroma and taste, originating from different sources including degradation of carbohydrates, amino acids, and lipids both during sourdough fermentation, as well as through caramelisation and Maillard reaction products during baking (Drapron and Molard, 1979; Schieberle 1989). Flavour of bread can be divided into two categories: crust flavour and crumb flavour. Crumb flavour mainly comes from sourdough fermentation while crust flavour is mainly produced during baking. Metabolism of LAB contributes to both crumb flavour and crust flavour. Fermentation by LAB produces flavour compounds such as 2- or 3-methylbutanal and acetic acid, which contribute to typical bread flavour. In addition, some metabolites produced by LAB, such as ornithine, contribute to crust flavour by conversion into flavour compounds through Maillard reaction during baking. Carbohydrates and amino acids offer the most flavour precursors in sourdough bread (Thiele et al, 2002; Martínez-Anaya 1996a, b). Generally more than 500 volatile compounds have been identified as volatile compounds in bread, mainly consisting of alcohols, aldehydes, ester, ketones, acids, pyrazines, pyrrolines, furans, hydrocarbons, and lactones (Nijssen et al, 1996; Kirchhoff and Schieberle, 2002). Studies on flavour compounds in sourdough have allowed reconstitution of bread flavour in vitro (Kirchhoff and Schieberle, 2002). Some characteristic flavour compounds produced during

sourdough fermentation are summarized in Table 1.1. Production of flavour compounds is strain-specific, depending on the metabolic pathway used by LAB during sourdough fermentation. Lactobacilli strains possess two pathways to utilize hexose: homo-fermentative pathway and hetero-fermentative pathway. Homo-fermentative lactobacilli metabolize hexoses via basic glycolysis and produce lactic acid as the main metabolic endproduct, whereas hetero-fermentative lactobacilli degrade hexoses via the pentose phosphate pathway into a strain-dependent ratio of lactic acid, acetic acid, CO_2 and ethanol (Figure 1.3) (Gänzle et al, 2007; Stolz et al, 1996). Depending on metabolism of hexose, lactobacilli strains can be divided into three groups: obligate homo-fermentative lactobacilli, facultative hetero-fermentative lactobacilli and obligate hetero-fermentative lactobacilli. Generally, homo-fermentative lactobacilli produce diacetyl and some carbonyls, whereas ethylacetate, certain alcohols and aldehydes, and acetaldehyde are produced by hetero-fermentative strains (Damiani et al, 1996).

1.2.2. Metabolism of carbohydrates in LAB and bread flavour.

Acetate, one of the most important characteristics of sourdough bread flavour, can be produced from metabolism of sugars by hetero-fermentative strains of lactic acid bacteria in sourdough in presence of electron acceptors such as fructose or O_2 (Stolz et al, 1995; Röcken et al, 1992). One exception is *Weissella* species, which are employed as starter cultures for both wheat and sorghum sourdough. This

Table 1.1. Flavour compounds in sourdough produced through metabolism in LAB (1: Czerny and Schieberle, 2002; 2: Kirchhoff and Schieberle, 2002; 3: Damiani et al, 1996; 4: Molimard and Spinnler, 1996; 5: Belitz et al, 2004).

Compound	Flavour	Substrates	Odor Threshold (mg L^{-1})	Amount (mg kg^{-1})	Reference
Acetic acid	Acetic-like, sour	Glucose		134-218	1
Ethanol	Alcohol	Glucose	100	330–1950	1,3,4,5
3-methylbutanal	malty	Leucine	0.0004	0.097 -0.153	1,2
3-methylbutanol		Leucine	0.25	15.2	2,5
3-methylbutanoic acid	sweaty	Leucine	0.740	2.58	1,2
2-methylbutanal	malty	Isoleucine	0.0037	0.03 -0.074	1
2-methylbutanol		Isoleucine			
2-methylbutanoic acid	sweaty	Isoleucine		0.939	1,2
2-methylpropanal		Valine	0.001	0.020	2,5
2-methylpropanol		Valine			
2-methylpropanoic acid		Valine			
Phenylacetaldehyde	Honey-like	Phenylalanine		0.183 -0.508	1
Phenylethanol		Phenylalanine			2
Phenylacetic acid	Honey-like	Phenylalanine		0.142-0.418	1,2
Diacetyl	butter	Aspartate	0.015	0.744	2
Acetoin	butter	Aspartate			
2-acetylpyrroline	Roastry, popcorn-like	Ornithine			2
butanoic acid	Sweaty			21.8	2
pentanoic acid	Sweaty			11.6	1,2
vanillin	Vanilla like		0.02	2.790	2,5
Glutamate	Umami	Glutamate			

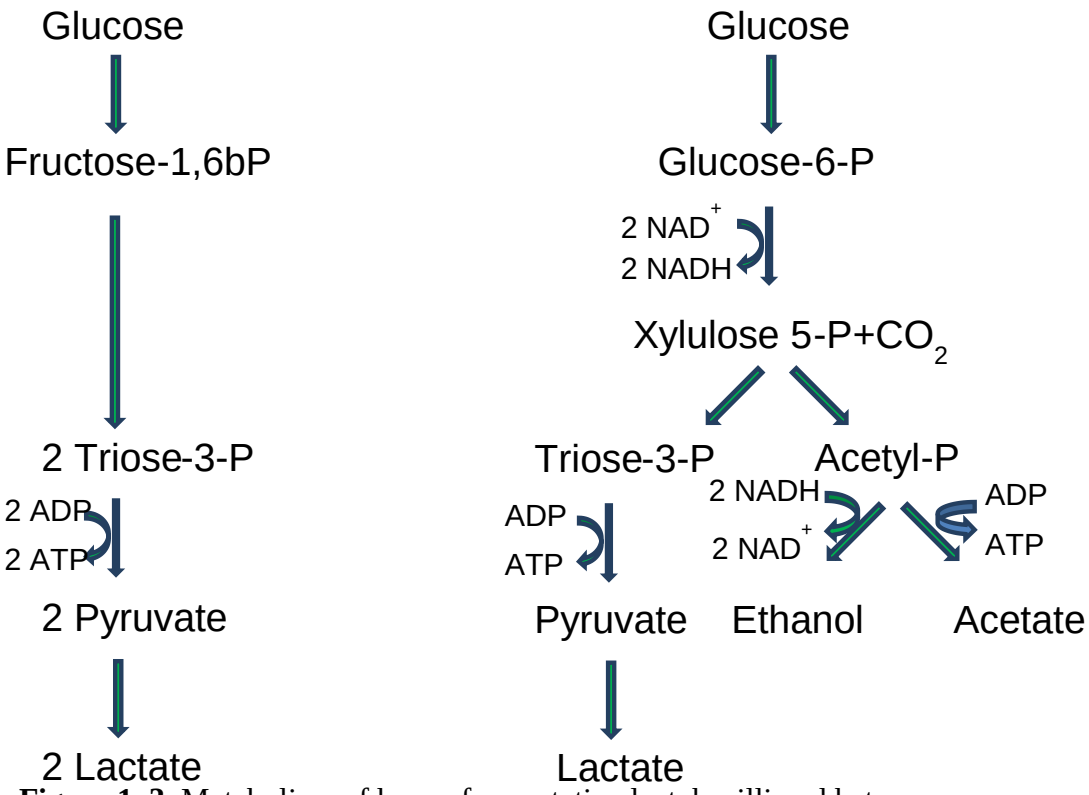

Figure 1. 3. Metabolism of homo-fermentative lactobacilli and hetero-fermentative lactobacilli (based on Gänzle et al, 2007).

genus utilizes sucrose to synthesize EPS and glucooligosaccharides but only very low concentration of acetate (Galle et al, 2010). Increased production of acetate improved the quality of the Italian traditional sourdough product Colomba (Vernocchi et al, 2004). Although a certain amount of acetic acid can enhance wheat bread flavour, a high concentration of acetate cause a pungent and unpleasant flavour (Molard et al, 1979; Molard and Chagnier, 1980; Salovaara and Valjakka, 1987; Lilja et al, 1993).

Production of diacetyl /acetoin can be also achieved during metabolism of carbohydrates in LAB. Diacetyl, which imparts a butter-like flavour, is an important flavour compound of the bread crumb (Hansen and Schieberle, 2005).

Compared to hetero-fermentative lactobacilli, production of diacetyl by homo-fermentative lactobacilli is much higher in both sourdough and bread (Damiani et al, 1996; Hansen et al, 1989; Lund et al, 1989; Hansen and Hansen, 1996). The amount of diacetyl produced is lower in bread than its corresponding sourdough (Hansen and Hansen, 1996; Lund et al, 1989). Acetoin is also a butter-like flavour compound, and is produced by *Lactococcus lactis* (Le Bars and Yvon, 2008). During sourdough fermentation, metabolism of glucose by LAB produces acetolactate via pyruvate, and then acetolactate is either reduced to acetoin through decarboxylase activity or chemically oxidized to diacetyl (Hugenholtz et al, 2000). Diacetyl can be further converted to acetoin in presence of acetoin/diacetyl reductase. Diacetyl formation during growth in sourdough was reported for strains of *L. plantarum*, *Lactobacillus farciminis, L. alimentarius,* and *Lactobacillus acidophilus*, with the maximum production identified in *L. alimentarius* (Damiani et al, 1996). In addition, cells extracts of *Leuconostoc lactis* can also form diacetyl in presence of pyruvate (Jordan et al, 1996). Conversion of pyruvate to acetolactate is catalyzed by acetolactate synthase (ALS). Characterization of ALS has been studied in *Lactococcus lactis*, which has an optimum pH range of 5.8-7.0 and an optimum temperature range of 38-40°C (Kisrieva et al, 2001). Diacetyl/acetoin was produced up to 1,400 mg L^{-1} by expression of ALS from *Lactococcus lactis* in *Lactobacillus casei* (Nadal et al, 2009).

1.2.3. Metabolism of amino acid in LAB and bread flavour.

The initial step of metabolism of amino acids by LAB involves the action of the enzymes aminotransferase, decarboxylase, lyase, or hydrogenase, as previously reviewed (Figures 1.4, 1.5, and 1.6) (Ardö 2006; Fernández and Zúñiga, 2006).

Aminotransferase activity

Metabolism of common amino acids can start with amino group transformation. During amino group transformation, the substrate amino acid has one amino group removed, and is then converted into the corresponding α-keto acids. Another amino group acceptor receives this amino group, and is converted into the corresponding amino acids (Figure 1.4). Alpha-ketoglutarate (α-KG) is used as a common and efficient amino group acceptor and its corresponding product is glutamate. Pyruvate and oxaloacetate have also been reported as amino group acceptors; however, the efficiency of α-KG is much higher (Yvon et al, 2000). Amino acid transformation is catalyzed by substrate-structure dependent aminotransferases, which have been identified in various LAB strains (Yvon and Rijnen, 2001; Thage et al, 2004). Amino acids relevant to flavour production in sourdough fermentation include the aromatic amino acid phenylalanine and tyrosine, and the branched-chain amino acids leucine, isoleucine and valine (Yvon and Rijnen, 2001; Thage et al, 2004). Both pleasant and unpleasant flavour compounds are produced by LAB through catabolism of amino acids (Urbach, 1993). Further catabolism of α-keto acids produces flavour compounds (Table 1.1). There are three metabolic pathways for further catabolism of α-keto acids in branched-chain amino acids, which are through hydroxyisocaproate

dehydrogenase, decarboxylation through α-oxoacid decarboxylase and decarboxylation to carboxylic acid (Yvon and Rijnen, 2001). The pathway to carboxylic acid has been studied in *Lactobacillus helveticus* and *Streptococcus thermophilus* (Helinck et al, 2004).

Metabolism of leucine, isoleucine, and valine through transamination produces various flavour compounds such as carboxylic acids including 2- or 3-methylbutanoic acid, aldehydes including 2- or 3-methylbutanal, and alcohols including 2- or 3-methylbutanol (Yvon and Rijnen, 2001; Marilley and Casey, 2004; Smit et al, 2005). However, only carboxylic acids are most commonly produced in sourdough. Increased concentrations of 3-methylbutanal after fermentation in both whole wheat sourdough and sourdough fermented with white wheat flour has been identified (Czerny and Schieberle, 2002). 3-Methylbutanal has malty and cheesy flavours; 3-methylbutanol has fresh cheese and alcoholic flavours; 3-methylbutanoic acid has rancid sweat, cheese and putrid flavours (Smit et al, 2005). Genes encoding branched-chain aminotransferase have high similarity among LAB and are characterized in *L. paracasei* and *L. plantarum* (Thage et al, 2004). A similar conversion rate has been reported for branched-chain aminotransferase from *L. paracasei* to convert isoleucine, leucine, with α-KG as amino group acceptor. This enzyme has an optimum pH and temperature of 7.3 and 43 °C (Thage et al, 2004; Nierop Groot and de Bont, 1998).

Metabolism of phenylalanine starts with transamination or decarboxylation. Only transamination of phenylalanine is flavour relevant. Decarboxylation of phenylalanine leads to production of the biogenic amine phenylethylamine (Yvon et al, 1997; Gao et al, 1997). Transamination of phenylalanine results in

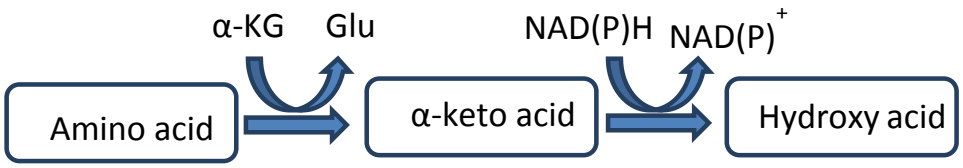

Figure 1. 4. Metabolism of amino acids by transamination (Based on Ardö 2006).

production of phenylpyruvate, which is then degraded to phenylactatealdehyde, a major flavour compound in both cheese and bread. Transamination of phenylalanine is catalyzed by the aromatic aminotransferase AraT, which has been well studied both biochemically and genetically in *Lactococcus lactis* strains (Yvon et al, 1997; Gao and Steele, 1998; Rijnen et al, 1999).

Deiminase and decarboxylase

In LAB**,** degradation of asparagine generally starts with conversion to aspartic acid (Asp) by asparaginases, with emission of ammonia. Asparaginases are particularly important for reducing production of the presumed carcinogen acrylamide during baking (Konings et al, 2007). Although some strains of LAB have been reported to utilize asparagine, asparaginase activity in *Lactobacillus* strains have not yet been reported (Williams et al, 2001). Further degradation of Asp by aspartate aminotransferase results in production of oxalacetate, which can be converted into diacetyl or acetoin as previously described (Ardö 2006).

Metabolism of glutamine also generates beneficial metabolites (Figure 1.5). Glutaminase converts glutamine to glutamate, which imparts umami flavour. Glutaminase has a two-fold activity: it can convert glutamine to glutamate and ammonia through hydrolytic activity, and also to theanine by glutamyl transfer activity. Both reactions enhance flavour (Nandakumar et al, 2003). Identification of glutaminase in sourdough fermentation has been reported in *L. sanfranciscensis* and *L. reuteri* (Vermeulen et al, 2007). Glutamine is one of the most abundant amino acids in proteins of wheat, and can comprise more than 20% of the total amino acids (Wieser, 2004). Glutamate can be further converted into GABA by decarboxylase activity, which has been applied in sourdough fermentation (Rizzello et al, 2008a).

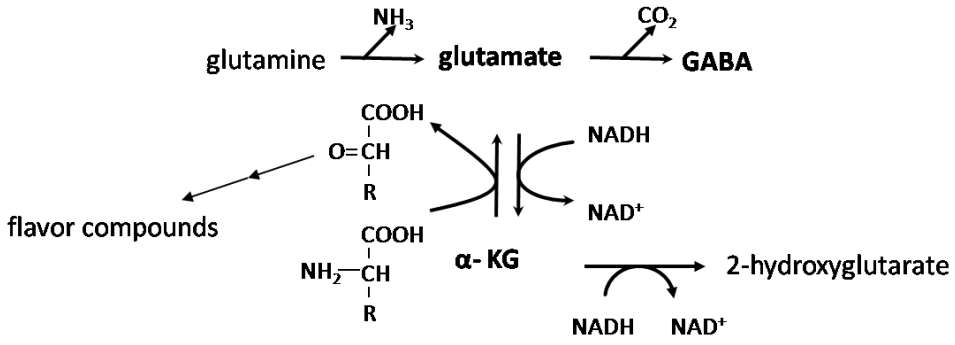

Figure 1. 5. Glutamate metabolism in lactobacilli. Beneficial metabolites include: Glutamate, umami taste; GABA, antihypertensive property; α- KG, cofactor for producing flavour compounds (Weingand-Ziadé et al, 2003; Tanous et al, 2005; Radler and Bröhl, 1984; Ueno et al, 1997).

Ornithine is a precursor compound of 2-acetyl-1-pyrroline, a key flavour compound of the wheat bread crust formed during baking (Schieberle and Grosch, 1985). Ornithine in wheat dough is derived from the yeast biomass or metabolism of arginine in lactobacilli. Several species of lactobacilli, e.g. *L.*

amylolyticus, *L. brevis*, *L. fermentum*, *L. frumenti*, *L. pontis*, *L. reuteri*, and *L. sakei* convert arginine to ornithine via the arginine deiminase (ADI) pathway (Thiele et al, 2002; Hammes and Hertel, 2003). ADI pathway includes ADI, catabolic ornithine transcarbamoylase (OTC) and carbamate kinase (CK), which has been characterized in lactobacilli (Arena et al, 2002).

Decarboxylation of amino acids such as histidine, tyrosine and ornithine can result in production of histamine, tyramine and diaminobutane, respectively. Those biogenic amines have become important health and safety concerns as they can cause toxic effects when consumed in large amounts (Shalaby 1996). Decarboxylation of histidine has been reported in many *Lactobacillus* strains including *L. sakei*, *L. bulgaricus*, and *L. acidophilus* (Dapkevicius et al, 2000; Chander et al, 1989; Bover-Cid and Holzapfel, 1999). Their presence in various foods has been studied, for more details see reviews (Suzzi and Gardini, 2003; Spano et al, 2010; Coton et al, 2010; Moreno-Arribas and Polo, 2008). Biogenic amines other than GABA have not been detected in sourdough fermentation (Stromeck et al, 2011).

Dehydrogenase activity

Glutamate produced during fermentation can be converted into α-KG and ammonia by glutamate dehydrogenase (Kieronczyk et al, 2003). Alpha-KG serves as amino group acceptor as mentioned above. This reaction can be either NAD^+ or $NADP^+$ dependent. Study with 156 LAB isolates for glutamate dehydrogenase activity using both NAD- and NADP-dependent methods showed that most isolates that have glutamate dehydrogenase activity have more NADP activity

than NAD activity (Fernández and Zúñiga, 2006). The gene encoding glutamate dehydrogenase has been characterized in *Lactococcus lactis* (Tanous et al, 2005).

Lyase activity

Cystathionine lyase (C-S lyase) plays an important role in formation of volatile sulfur compounds from methionine, which is particularly important during cheese ripening (Hanniffy et al, 2009). Both cystathionine β- and γ-lyases convert methionine to methanethiol and were characterized in *Lactococcus lactis* (Alting et al, 1995; Bruinenberg et al, 1997). Mechanism of C-S lyase involved in production of volatile sulfur compounds is not well understood yet, but it was suggested that C-S lyase was involved in both conversion of cystathionine and methionine to volatile sulfur compounds (Lee et al, 2007). A number of *Lactobacillus* strains were reported to possess C-S lyase activity including *L. plantarum*, *L. casei*, *L. fermentum*, and *L. reuteri* (Hanniffy et al, 2009; De Angelis et al, 2002).

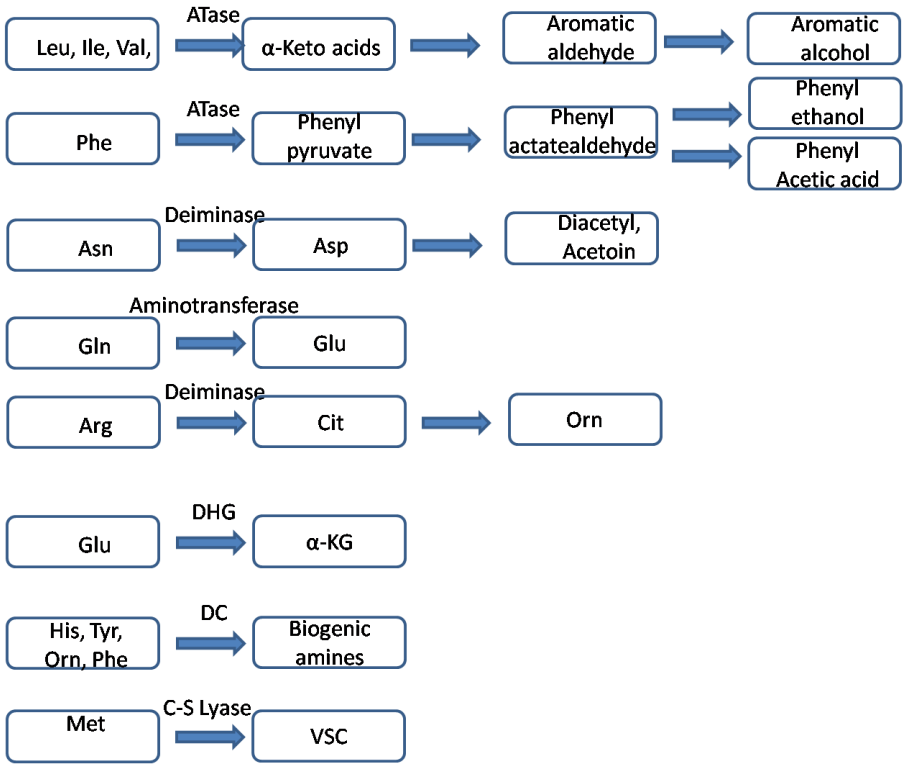

Figure 1. 6. Flavour compounds related to amino acid metabolism in LAB. VSC: volatile sulfur compounds.

1.3. Metabolism in LAB and bread texture.

1.3.1. Introduction of metabolism in LAB and bread texture.

Texture can generally be defined as "group of physical characteristics that are sensed by the feeling of touch, are related to the deformation, disintegration, and flow of the food under the application of a force and are measured objectively by functions of force, time and distance'' (Bourne 1982; Szczesniak 1987). During sourdough fermentation, acidification by LAB and exopolysacchride (EPS) production impacts bread texture. In addition to microbial enzymatic reactions during sourdough fermentation, physical properties of dough also change (Spicher 1995).

1.3.2. Acidification

Acidification in rye sourdough plays an important role by solubilizing pentosan, which determines rye bread volume (Korakli et al, 2001). In wheat sourdough fermentation, the major proteinase activity comes from aspartate class proteinase (Loponen et al, 2004). Optimum pH of wheat flour proteases is around 4 (Kawamura and Yonezawa, 1982). Since gases produced during leavening are held by the gluten network in wheat sourdough, decreasing the pH by acidification plays an important role in changing proteinase activity. Both increased and decreased volumes of bread resulting from acidification by LAB have been reported, depending on conditions and extent of fermentation. More elastic behavior of acidified wheat doughs have been identified under ideal

conditions (Wehrle et al, 1997). Bread volume was increased by using sourdough acidified by LAB compared to chemically acidified doughs (Clarke et al, 2002; Corsetti et al, 2000). Addition of 20% sourdough improved crumb softness in wheat bread (Crowley et al, 2002). Decreases in elasticity and viscosity have been reported in both chemically acidified and biologically acidified wheat sourdough. Biological acidification was conducted by fermentation of *L. brevis* for 24 hours, and later images from microscopy illustrated that gluten network became more amorphous structure due to fermentation (Clarke et al, 2004).

1.3.3. EPS produced by LAB and bread texture

EPS are extracellular sugar polymers that have strong ability to absorb water and are thus an ideal candidate to improve bread texture, especially gluten free bread (Arendt et al, 2007). Increased interest towards EPS produced by LAB is due to the requirement of replacing more expensive hydrocolloids and plant polysaccharides. Depending on composition of subunits, EPS can be divided into two classes: homopolysaccharides (HoPS) and heteropolysaccharides (HePS). HoPS are composed of the same repeating monosaccharide in different linkages and can be subdivided into two groups: glucans composed by glucose and fructans composed of fructose. Glucans and fructans are produced through glycosyltransferase activity with sucrose as glycosyl donor (Tieking and Gänzle, 2005). Glucans with α- 1→3, 4, 6 linkage are commonly found and production of dextran, amylose, mutan, alternan, reuterran *etc* are identified in various *Lactobacillus* species (Korakli and Vogel, 2006). Reuteran, dextran, and levan

were applied in baking to improve bread quality (Brandt et al, 2003; Tieking et al, 2003). Fructan with the β-2→1, 6 linkage is the most common such as inulin and levan (Tieking and Gänzle, 2005). HePS are produced by intracellular glycosyltransferases (De Vuyst et al, 2001). Individual sugar units produced by LAB are mainly glucose, galactose, fructose, rhamnose, and their ratio of composition are dependent on the structure of EPS (De Vuyst and Degeest, 1999; van Hijum et al, 2006). In contrast to HoPS, production of HePS does not require addition of sucrose and thus leads to lower production of acetate. To date, a variety of EPS have been identified in LAB, and most of EPS responsible for improving bread texture are HoPS. HePS are usually produced less than 1 g L^{-1} during sourdough fermentation thus can not play an important role (De Vuyst and Degeest, 1999; Laws and Marshall, 2001; De Vuyst et al, 2001). Galle et al (2010) reported that HePS produced by *Lactobacillus buchneri* resulted in decreased resistance to deformation in sorghum sourdough associated with gluten free bread. Although similar results could not be obtained from wheat sourdough, the decreased resistance to deformation could potentially increase bread volume and elasticity of sorghum sourdough (Hüttner et al, 2010; Renzetti and Arendt, 2009)

EPS produced by LAB has been applied in sourdough fermentation to improve dough rheology and bread texture (Decock and Capelle, 2005). Impact of fructan production on improvement of dough rheology and bread texture has been investigated in *L. sanfranciscensis* (Brandt 2001; Korakli et al, 2000). HoPS produced by *L. plantarum* reach around 7.5 g L^{-1} after 6 days incubation in MRS media, and production of HoPS increased wheat bread volume by around 20%

and decreased bread firmness by around 50% compared to the EPS-negative fermentation control (Di Cagno et al, 2006). In situ production of dextran by obligately heterofermentative *Weissella confusa* was examined in wheat sourdough. Up to 16 g kg^{-1} dry weight dextran was detected upon addition of sucrose, which significantly increased the viscosity of sourdough. Bread containing dextran has mildly acidic taste, but improved bread volume by around 10% and more than 20% on crumb softness during storage for 6 days (Katina et al, 2009). Addition of 1% in situ-produced dextran improved bread volume by 32%, and wheat sourdough bread containing dextran showed more than 20% less hardness than bread without sourdough after 2 weeks conservation (Lacaze et al, 2007). Dextran produced by *Weissella cibaria* increased softness of sorghum bread (Schwab et al, 2008). Addition of EPS produced by *Lactobacillus curvatus* improved wheat bread volume by more than 20% and decreased the firmness by more than 50% (Minervini et al, 2010). HoPS produced in situ have been reported to be more effective than externally added (Kaditzky et al, 2008; Katina et al, 2009). However, hetero-fermentative LAB metabolize sucrose as electron acceptor, with the production of acetate (Korakli et al, 2001; Wisselink et al, 2002). Higher levels of acetic acid negatively affect taste and volume of bread (Kaditzky and Vogel, 2008).

In situ EPS production is challenged by control of fermentation conditions during sourdough fermentation. Enzymes responsible for EPS production can be affected by various conditions such as temperature, pH, availability of acceptor molecules, and dough yield (Kaditzky and Vogel 2008; Kaditzky et al, 2008). Increased

production of EPS has been reported in *L. reuteri* under constant pH of 4.7, additional sucrose, and higher dough yield (Kaditzky and Vogel, 2008). Up to 50 g kg^{-1} of wet medium EPS has been reported with additional glucose and sucrose (Minervini et al, 2010).

1.4. Metabolism in LAB and bread shelf life.

1.4.1. Introduction of metabolism in LAB and bread shelf life.

Bread keeps fresh only for several hours due to high temperature of baking. After that, various changes occur in both crumb and crust including microbial growth and physical changes. Those changes can be generally classified into two categories: bread staling and bread spoilage. A general term accepted for bread staling is proposed by Bechtel et al, (1953), who defines staling as:"a term which indicates decreasing consumer acceptance of bakery products caused by changes in crumb other than those resulting from the action of spoilage organisms". Staling of bread involves moving of moisture from crumb to crust and thus results in harder crumb and softer crust (Arendt 2007), which has been reviewed by Gray and Bemiller in 2003. Retrogradation has been reported as the main reason for staling (Zobel and Kulp, 1996).

Bread staling is more rapid in bread lacking a gluten network, such as gluten free bread, than in breads containing gluten (Moore et al, 2004). Use of sourdough in bread resulted in delayed spoilage, and the effect is independent of acidification (Arendt et al, 2007). Additionally, EPS produced by sourdough fermentation also

prevented bread staling. Sourdough fermented by *L. plantarum* delayed bread staling compared with those prepared from chemically acidified dough during storage (Moore et al, 2008). When bread dough leavened with yeast and chemically acidified dough followed by leavening with yeast are compared with sourdough, it was found that only sourdough fermentation can delay bread staling. This effect is even greater when pentosans was added (Corsetti et al, 2000). Incorporation of 20% sourdough in wheat bread showed delayed firming (Crowley et al, 2002).

In addition to bread staling, fungi and bacterial growth can also lead to bread spoilage. Most bread spoilage is caused by fungal growth. Bacterial spoilage is mainly caused by rope forming *Bacillus* sp. such as *Bacillus subtilis* and *Bacillus licheniformis* (Kirschner and Von Holy, 1989). Traditionally sourdough has been used to prevent bread spoilage. *Bacillus* spoilage can be prevented by low pH. Acidification by addition of 20% sourdough fermented by *L. plantarum* and *L. alimentarius* prevented visual growth of both *B. subtilis and B. licheniformis* (Mentes et al, 2007). Reutericyclin is a highly hydrophobic tetramic acid produced by *L. reuteri* which has broad inhibitory spectrum against spoilage bacteria and less effect in yeast and fungi as reviewed by Gänzle (2004). The minimum inhibitory concentration (MIC) of reutericyclin was reported as 0.13-0.31 mg L^{-1} against common rope spoilage *B. substilis* strains at neutral pH (Gänzle et al, 2000). Concentrations of reutericyclin produced in wheat sourdough reached 5 mg kg^{-1} (Gänzle and Vogel, 2002).

The positive effect of LAB on both bread staling and spoilage has been investigated, which involves antifungal substances produced through metabolism of LAB and acidification on rheological change by LAB fermentation (Axford et al, 1968; Maleki et al, 1980).

1.4.2. Antifungal activity of LAB.

Growth of molds on the surface of bread is one of the most important causes of bread spoilage. Fungal spoilage results in waste of approximately 5-10% of the world's food production (Pitt and Hocking, 1999). The most common fungi isolated from spoiled bread are *Penicillum* spp, which could account for more than 90% spoilage in Northern Ireland (Legan 1993). *Penicillium roquefortii* has been reported as one of the most resistant bread spoilage mold, but there are few applications reported for inhibition of this organism (Lind et al, 2005; Valerio et al, 2009; Ryan et al, 2011). Many substances produced by LAB, including acetate, propionic acid, phenyallactate, diacetyl, reuterin, and dicyclic peptides, have been proven to possess antifungal activity (Lind et al, 2005; Jay 1982; Ryan et al, 2008; Ström et al, 2002; Gerez et al, 2010). Recently, *L. reuteri* showed the strongest inhibitory effect against *Penicillium expansum* among the 165 LAB tested (Guo et al, 2011).

Lactate, acetate, propionate

Lactate, acetate, and propionate have pKa value of 3.86, 4.76, 4.87, respectively, resulting in 6.8%, 37%, 43% in undissociated form around pH 5.0 (Lind et al, 2005). Lactate is produced from sugar fermentation by both homo-fermentative

and hetero-fermentative LAB. A high concentration of lactic acid is required to exert an antifungal effect, with MIC higher than 500 mM towards most common bread spoilage fungi including *Penicillium*, *Aspergillus* and *Fursarium* (Lind et al, 2005). Compared to lactic acid, acetate has more effect on fungi but high amount of acetate inhibit the activity of baker's yeast. Classic weak acid theory proposes that undissociated weak acids pass through cytoplasm membrane and dissociate in the cytoplasm. The resulting acidification in the cytoplasm impacts on cell function. Propionic acid has higher antifungal activity, with an MIC of 20-50 mM required to inhibit five common spoilage molds including *P. roquefortii*, compared to 30-120 mM for acetate at pH 5.0 (Lind et al, 2005). Additionally, both acetic acid and propionic acid have pungent odors, with odor thresholds of 22-54 and 40 mg L^{-1}, respectively, and both are part of the characteristic flavour of Camembert cheese (Brennand et al, 1989; Molimard and Spinnler, 1996). EU directive 95/2/CE allows up to 0.3% (wt/wt) addition of calcium propionate in bakery products. The mechanism of the antifungal activity of propionic acid relies on reduction of cytoplasmic pH as well as conversion of propionic acid to propionyl-CoA, which interferes with metabolism of glucose in mold by inhibiting pyruvate dehydrogenase (Brock and Buckel, 2004). At pH 4.5, 25 °C, and a_W of 0.85, 0.03% propionate significantly inhibits the growth of *Aspergillus* spp. and *Penicillium corylophilum* (Guynot et al, 2005). Moreover, there has been no reported effect of propionate on baker's yeast activity, which makes propionate the best potential preservative among the three. Sorbic acid and benzoic acid have also been often added into baked goods; however, the pKa of benzoic acid is

around 4.2 and is thus less effective than propionate, while sorbic acid has an adverse effect on dough rheology and inhibits the growth of yeast (Legan 1993).

Diacetyl

Diacteyl has a broad inhibitory spectrum against spoilage molds and yeast. At pH 5.0, 300 mg L^{-1} diacetyl completely inhibits the growth of six molds, including *Penicillium* and *Aspergillus*, although activity of *Saccharomyces cerevisiae* has also been inhibited (Jay 1982); however, the high concentration of diacetyl required for an optimal inhibitory effect changes the flavour of bread (Piard and Desmazeaud, 1991). Up to 744 μg kg^{-1} dry wt diacetyl was produced in rye sourdough using a commercial starter culture (Kirchhoff and Schieberle, 2002). Diacetyl has quite an intensive odor; however, the level required for inhibition of fungi is much higher than acceptable levels of odor.

Phenyllactate and hydroxyl-fatty acids

Phenyllactate shows intensive antifungal activity against 14 species of spoilage fungi including *Aspergillus*, *Penicillium* and *Fusarium* (Lavermicocca et al, 2003). Condidial germination assay with *L. plantarum* showed complete inhibition of molds from 5 genera including *P. roquefortii* (Lavermicocca et al, 2000). Phenyllactic acid and 4-hydroxyphenyllactic acids were later identified as the responsible antifungal compounds. Bread baked with sourdough fermented with *L. plantarum* showed delayed spoilage up to one week after inoculation with *A. niger*. More than 90% of growth inhibition has been reported for all 23 fungal strains tested, with two days delay in growth of *P. roquefortii* (Lavermicocca et al, 2003). Moreover, production of 33 mg kg^{-1} dough of phenyllactate was reported

in *L. plantarum* in sourdough, with the highest production observed during logarithmic growth (Ryan et al, 2008). However, MIC of phenyllactate against common fungi isolated from bread is more than 3 g L^{-1}, but currently only around 33 mg kg^{-1} sourdough can be produced, thus phenyllactate produced is not able to play an important role (Ryan et al, 2009).

Cyclic dipeptides

2, 5-diketopiperazines such as cis-cyclo (L-Leu-L-Pro) and cis-cyclo (L-Phe-L-Pro) are two cyclic dipeptides, which have been reported to contribute to antifungal activity of *L. plantarum* (Niku-Paavola et al, 1999; Ström et al, 2002). Cyclo (Leu–Pro) and cyclo (Phe–Pro) have been shown to contribute to antifungal activity against *Aspergillus niger* and *Fusarium* spp. (Dal Bello et al, 2007). In 2010, Cyclo (Leu–Leu) isolated from *L. plantarum* in kimchi shown broad spectrum inhibition against fungi including *Penicillium*, *Aspergillus*, *Epicoccum*, and *Cladosporium*; however, since the purity of the compounds was very low, further investigation is needed. It is not yet clear whether or not cyclic dipeptides reported in sourdough results from metabolic activities of LAB or acidification (Ryan et al, 2009). Recently, cyclic dipeptides from wheat sourdough fermented by *L. amylovorus* were reported to contribute to the antifungal activity (Ryan et al, 2011). The MIC of 2, 5-diketopiperazines against common bread spoilage fungi is approximately 20 mg g^{-1} (Ström et al, 2002; Ryan et al, 2008). The concentration currently produced during sourdough fermentation was reported to be 1000 times lower. Additionally, cyclic dipeptides possess bitter taste, which limits their application (Ryan et al, 2009).

Hydroxyl-fatty acids

3-(R)-hydroxydecanoic acid, 3-hydroxy-5-cis-dodecenoic acid, 3-(R)-hydroxydodecanoic acid and 3-(R)-hydroxytetradecanoic acid from *L. plantarum* have been reported to have strong antifungal acidity at 10-100 mg L^{-1} against spoilage fungi including *P. roquefortii*. However, the concentration produced by *L. plantarum* was 10-100 times lower than the MIC, and thus could not play an important role in wheat sourdough (Sjoegren et al, 2003).

Reuterin

Reuterin is an intermediate product of glycerol metabolism in *L. reuteri*. Reuterin showed strong antifungal acitivity against moulds including *Aspergillus* and *Fusarium* species; however, it also inhibited *Saccharomyces* (Talarico et al, 1988). Production of reuterin in sourdough has not yet been reported. However, 1, 3-propanediol was produced in sorghum sourdough and thus offers the possibility of reuterin production (Sekwati-Monang, 2011).

1.5. Aim of the research.

Fermentation of propionate has only been studied in silage fermentation, and its application in sourdough fermentation is still lacking. As an important preservative in sourdough, the effect of propionate is much higher than that of lactate and acetate, and biologically produced propionate can replace calcium propionate.

L. sanfranciscensis and *L. reuteri* are two of the most commonly isolated strains from Type I and Type II sourdough, respectively. Study of α-KG has most been conducted in cheese fermentation, and its study in *Lactobacillus* strains has been ignored to date. Understanding the metabolic pathway of α-KG in *Lactobacillus* strains will greatly help production of flavour compounds during wheat sourdough fermentation as α-KG is the most common amino group acceptor for further degradation of amino acids in LAB to produce flavour compounds.

Glutamine metabolism is of great importance for wheat, rye and barley fermentations as glutamine compose of more than 20% of amino acids in those cereals (Wieser, 2004). Glutaminase in lactobacilli converts glutamine to glutamate. Glutaminase activity contributes to acid resistance of starter culture in sourdough. Genomic characterization of glutaminase has been conducted for *Bacillus* spp. and *Escherichia coli* but not for *Lactobacillus* strains. Understanding fundamental knowledge of glutamine metabolism will allow fast screening of starter culture with desired metabolites from glutamine. Several acid resistance systems have been found in *L. reuteri*, but their efficiency has not been compared yet.

The goal of this research was to develop a better understanding of the ecological role of metabolic pathways in LAB used in sourdough. Specific aims include:

I. Enhanced production of propionate by cofermentation of *L. buchneri* and *L. diolivorans* in sourdough fermentation;

II. Characterization of the metabolic pathway of α-KG in *L. sanfranciscensis* and *L. reuteri* during sourdough fermentation;

III. Characterization of three glutaminases in *L. reuteri* 100-23 and study comparative effect of glutaminases with ADI and GAD pathway in that strain.

Strain of *L. buchneri* used was isolated from sorghum sourdough, and *L. diolivorans* used is chosen from commercial type strain from silage due to their ability to convert lactic acid to 1, 2-propanediol and convert 1, 2-propanediol to propionic acid, respectively. Strains of *L. sanfranciscensis* and *L. reuteri* used are currently employed by industry for sourdough fermentation. Strain of *L. reuteri* 100-23 was used as a model microorganism to study effect of glutaminase in acid resistance due to the availability of genome information.

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Chapter 2: Propionic acid production by cofermentation of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough

A version of this chapter has been published. Zhang, C, M. J. Brandt, C. Schwab, and M. G. Gänzle. 2010. Propionic acid production by cofermentation of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough. Food Microbiology, 27: 390-395.

2.1. Introduction.

Growth of molds on the surface of bread is considered one of the most important causes of bread spoilage. Most molds isolated from spoiled bread are *Penicillum* spp. (Legan 1993). To prevent bread spoilage, food preservatives such as lactate, acetate and propionate are added to inhibit growth of molds and thus improve food safety as well as extend shelf-life (Gould 1996). The antifungal activity of propionate is higher than that of lactate and acetate. In addition to the effect of propionic acid on the cytoplasmic pH, propionate is converted to propionyl-CoA, which inhibits pyruvate dehydrogenase and thus inhibited metabolism of glucose in mold (Brock and Buckel 2004). Propionate at a level of 0.03% was effective in inhibiting growth of *Aspergillus* spp. and *Penicillium corylophilum* at pH4.5, 25°C, and an a_W of 0.85 (Guynot et al, 2005). At pH 5, the minimum inhibitory concentration of propionate for five fungi, including *P. roquefortii*, was around 50 mM (Lind et al, 2005).

Sourdough fermented with heterofermentative lactic acid bacteria accumulates lactate and acetate with limited antifungal activity. Specific strains of lactobacilli produce antifungal compounds such as phenyllactate, hydroxyl-fatty acids, and cyclic dipeptides (Schnürer and Magnusson 2005). However, only few lactobacilli were evaluated with respect to their antifungal effect in baking applications (Lavermicocca et al, 2000; Ryan et al, 2008, 2009).

Propionate formation by *L. buchneri* and *L. diolivorans* during growth in silage was suggested to contribute to preservation (Driehuis et al, 1999; Krooneman et

al, 2002). Lactate is converted into 1, 2-propanediol by *L. buchneri* (Figure 2.1, Oude Elferink et al, 2001), and 1, 2-propanediol is further converted into propionate by *L. diolivorans* (Figure 2.1, Krooneman et al, 2002). Concentrations of propionate and acetate that are accumulated by co-cultures of *L. buchneri* and *L. diolivorans* have to date not been characterized in either food or feed fermentations. Nevertheless, propionate formation by lactobacilli may be exploited for bread preservation.

It was the aim of this project to characterize lactate conversion to propionate by cofermentation of *L. buchneri* and *L. diolivorans* during sourdough fermentation, and to determine whether the use of propionate-producing cultures can replace the addition of propionate as a preservative.

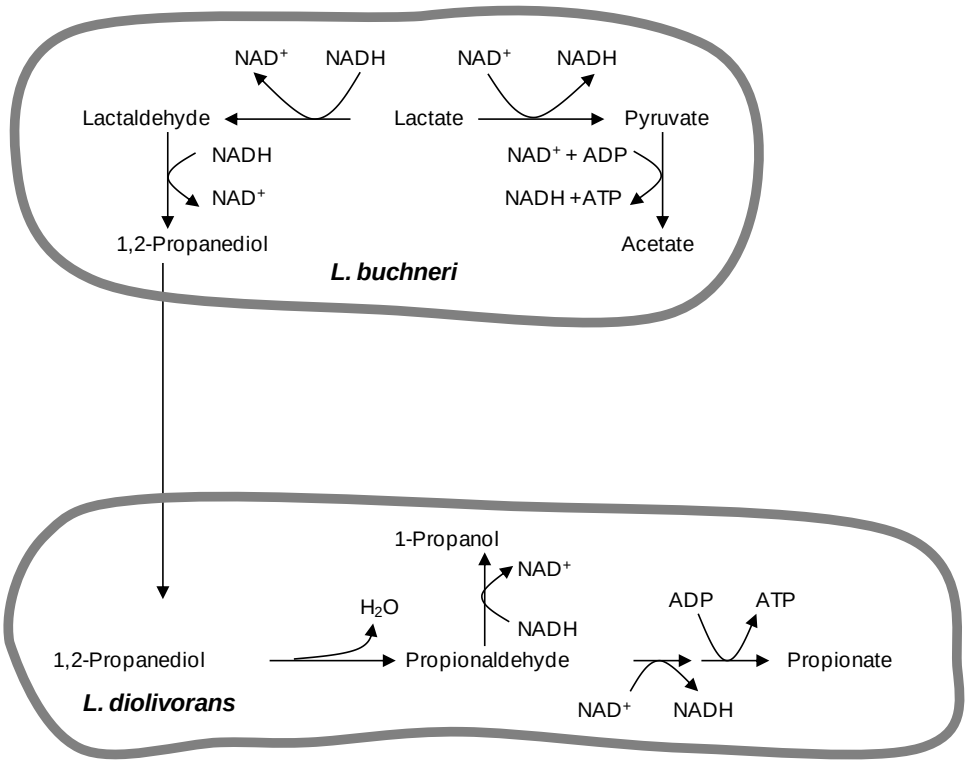

Figure 2.1. Metabolic pathway for conversion of lactate to propionate in cofermentation of *L. buchneri* and *L. diolivorans* (Oude Elferink et al, 2001; Sriramulu et al, 2008).

2.2. Methods.

2.2.1. Strains, culture and growth conditions.

L. diolivorans DSM14421 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). *L. buchneri* FUA 3252 was isolated from ting, a fermented sorghum product in Botswana (Sekwati-Monang and Gänzle, 2007). *L. buchneri* and *L. diolivorans* were grown in modified MRS (mMRS) containing 0.5% beef extract, 0.5% yeast extract, 1% peptone, 0.3% ammonium chloride, 0.4% K_2HPO_4, 0.26% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 0.005% $MnSO_4.4H_2O$, 0.1% Tween 80, 0.05% L-cysteine hydrochloride, and 0.0002% each of vitamin B1, B2, B6, B5, B12, and B9. Maltose, fructose, lactate and 1, 2 propanediol were added after autoclaving as described in the following sections. The pH of the media was adjusted to 5.7 and 6.0 for *L. buchneri* and for *L. diolivorans*, respectively.

2.2.2. Mold isolation, characterization and spores preparation.

One and three molds were isolated from blue mold cheese and bread, respectively. The bread was obtained in a local supermarket and stored until mold growth was visible. For isolation, molds grown on blue mold cheese and bread were streaked on meat extract agar medium and incubated for one week. Pure colonies were obtained by repeated streaking on meat extract agar.

Molds were identified based on morphology on both meat extract agar and Czapek agar (0.3% KNO_3, 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.05% NaCl,

0.001% $FeSO_4.7H_2O$, 3% sucrose, 1.5% agar). The blue mold cheese isolate was identified as *P. roquefortii* Thom. Fungal bread isolates were identified as *A. clavatus* Desmazieres, *Cladosporium* spp. and *Mortierella* spp. (Barnett and Hunter 1998; Ramirez 1982).

To obtain spores, molds were inoculated into meat extract agar and incubated for two weeks. Conidiospores were collected by immersing in physiological solution containing 0.85% NaCl and 2% peptone, filtered with Whatman No.1 filter paper and transferred into screwed tubes with 50% glycerol and stored at -80°C. Conidiospore counts in suspension were measured by serial dilution and spread plating.

2.2.3. Conversion of lactate to 1, 2-propanediol by *L. buchneri* in mMRS and conversion of 1, 2 propanediol to propionate by *L. diolivorans*.

The conversion of lactate and 1, 2-propanediol was initially analysed using single cultures of *L. buchneri* or *L. diolivorans*. Overnight cultures of *L. buchneri* were washed once in mMRS and five µL washed cells were used to inoculate 1 mL mMRS, pH 5.7, with additional 50 mM lactate and/or 5 mM maltose and/or 15 mM fructose. Cultures were incubated for 7 days at pH 5.7 and 30 °C. Overnight cultures of *L. diolivorans* were washed once in mMRS and five µL washed cells were used to inoculate 1 mL mMRS, pH 5.5, containing 50 mM lactate and/or 5 mM maltose and/or 15 mM fructose. Cultures were incubated at 30 °C for five days. Metabolites were analyzed using HPLC as described below. Experiments were performed in duplicate.

2.2.4. Cofermentation of *L. buchneri* and *L. diolivorans* in mMRS medium. Overnight cultures of *L. buchneri* and *L. diolivorans* were inoculated in mMRS medium containing either 10 mM maltose and 45 mM lactate (mMRSA), or 23 mM maltose, 11.5 mM fructose and 45 mM lactose (mMRSB) at equal optical densities (OD_{600} = 0.014). The pH was adjusted to pH 5.5. Cultures were incubated at 30 °C for 7 days. Metabolites were analyzed using HPLC as described below. Experiments were performed in duplicate.

2.2.5. Cofermentation in *L. buchneri* and *L. diolivorans* in sourdough. Sourdoughs with a dough yield of 200 were prepared using wheat flours Type 550 (white wheat flour), Type 1050 (medium ash content) and whole wheat flour. Overnight cultures of *L. buchneri* and *L. diolivorans* were inoculated at a cell density of 10^7 cfu g^{-1}. Sourdoughs were fermented at 30 °C for 14 days to ensure the formation of sufficient amounts of propionic acid. Samples were taken during the fermentation period to analyse cell counts, pH, and the concentration of metabolites. Colony morphology was determined to ensure that the two strains used as inoculum dominated the fermentation microflora throughout the fermentation, and to selectively enumerate *L. buchneri* and *L. diolivorans*. At 14 days of fermentation, only the original inoculants *L. buchneri* and *L. diolivorans* were recovered from the sourdoughs excluding the occurrence of microbial contamination. Metabolites were prepared and analyzed using HPLC as described below.

2.2.6. Analysis of metabolites with High Performance Liquid Chromatography (HPLC).

Organic acids, alcohols, and sugars were determined by HPLC with an Aminex HPX-87 column (300 mm x 7.8 mm, Biorad, USA) at a temperature of 80 °C and a flow rate of 0.4 mL min^{-1} with 5 mM H_2SO_4 as the eluent. The injection volume was 10 µl. Refractive index detector and UV detector (210 nm) were used for detection. Concentration of maltose, fructose, lactate, acetate, ethanol, 1, 2-propanediol, propionate, 1-propanol were determined using external standards. For sample preparations, 3.7% perchloric acid were added to supernatants of fermentation liquor or sourdough and incubated at 4˚C overnight. Precipitated protein was removed by centrifugation.

2.2.7. Baking experiments.

Sourdough was prepared using wheat flour Type 1050 and fermented with *L. buchneri* and *L. diolivorans* for 14 days as described above. Bread dough was prepared as summarized in Table 2.1 with addition of 5%, 10% or 20% of sourdough. For comparison, breads were prepared containing 10% one-stage BRS (Böcker Reinzucht Sauerteig Rye, fermented for 18 hours at 28 °C) or 10% one-stage BRS and 0.1% acetate (BRS-Ac). For control, breads were also prepared from straight dough and from straight dough with the addition of 0.4% Ca-propionate. Ingredients were mixed in a spiral kneader (SPA 12-4, Diosna, Osnabrück, Germany) for five min, shaped after 15 min dough resting time, at 25-26 °C and proofed for 50 - 60 min at 32 °C and 85 % r.m in pans. Proofing time

for the dough prepared with 20% addition of sourdough fermented with *L. buchneri* and *L. diolivorans* was extended to 240 min because of obvious inhibition of yeast activity. Bread was baked in a multi-deck oven set at a temperature of 220 °C (upper plate) and 210 °C (lower plate) for 45 minutes.

2.2.8. Antifungal activity.

Antifungal activity was determined according to Dal Bello et al, (2007) with slight modifications. Bread was cut into 1.5 cm wide slices. A ten fold dilution of mold stock was applied to both sides of the bread slices by spraying five spots on each side. Around 4.8×10^3, 8×10^2, 8×10^2, and 8×10^1 cfu conidiospores of *A. clavatus* Desmazieres, *Cladosporium* spp., *Mortierella* spp., and *P. roquefortii* Thom were sprayed to each spot of bread slices, respectively. Bread slices were wrapped in oxygen permeable plastic bags and stored at room temperature in a temperature controlled room. Mold growth was monitored daily for a duration of 12 days. Growth was recorded by counting the number of the total of 10 spots per slice that had visible growth of molds. A sample was considered spoiled if growth was visible on one of the 10 spots.

Table 2. 1. Ingredients of sourdough bread for baking [g].

	Recipe [g]						
Ingredients	**Control**	**5% SD**	**10% SD**	**20% SD**	**10% BRS**	**10% BRS – Ac**	**Propionate**
Wheat flour 1050	2000	1900	1800	1600	1800	1800	2000
experimental sourdough[a)]	0	180	360	720	0	0	0
BRS[b)]	0	0	0	0	360	360	0
Water	1200	1120	1040	840	1040	1008	1200
Yeast	40	40	40	40	40	40	40
Salt	36	36	36	36	36	36	36
Acetate	0	0	0	0	0	32	0
Ca-propionate	0	0	0	0	0	0	8

[a)] Wheat 1050 sourdough was fermented with *L. buchneri* and *L. diolivorans* for 14 days at 30 ºC. 5%, 10%, or 20% of the experimental sourdough was added to bread dough.
[b)] BRS, Böcker Reinzucht Sauerteig Rye, fermented for 18 hours at 28 °C.
[b)] BRS-Ac, Böcker Reinzucht Sauerteig Rye, fermented for 18 hours at 28 °C with 0.1% acetate.

2.3. Results.

2.3.1. Metabolism of *L. buchneri* and *L. diolivorans* in single and cofermentation in mMRS.

L. buchneri produced 1, 2-propanediol during growth in mMRS (Table 2.2). In the presence of maltose, a higher concentration of 1, 2-propanediol was synthesized compared to growth in the absence of maltose. The highest concentration of 1, 2-propanediol was obtained when *L. buchneri* was grown in presence of maltose and lactate. The addition of fructose increased the formation of lactate, acetate and ethanol but reduced formation of 1, 2-propanediol. *L. diolivorans* converted 1, 2-propanediol into propionate (Table 2.3). Highest concentrations of propionate were formed when *L. diolivorans* was grown with 1, 2-propanediol as sole carbon source. In absence of 1, 2-propanediol, production of propionate was very low. *L. diolivorans* synthesized higher amounts of propionate in mMRS with maltose and 1, 2-propanediol compared to mMRS with maltose, fructose and 1, 2-propanediol. However, less lactate, acetate and ethanol was produced.

Propionate formation during cofermentation of *L. buchneri* and *L. diolivorans* was observed only in mMRSB and resulted in the production of lactate, 43.6±2.3 mM; acetate, 28.5±1.5 mM; ethanol, 32.0±3.6 mM; propionate, 5.4±0.2 mM; and 1-propanol, 4.9±0.5 mM.

Table 2. 2. Change of metabolite concentration after fermentation by *L. buchneri* in mMRS for 7 days at 30 °C.

	Substrates [mM]			**Metabolites [mM]**[a)]						
	Maltose [5 mM]	**Lactate [50 mM]**	**Fructose [15 mM]**	**Maltose**	**Fructose**	**Mannitol**	**Lactate**	**Acetate**	**Ethanol**	**1,2-POH**
Δ[a)] **metabolite concentration**	+	+	+	-4.3±0.1	-15.3±0.0	9.3±0.6	7.9±0.1	11.8±0.8	12.6±1.4	0.8±0.2
	+	+	-	-4.4±0.1	-	-	-0.8±0.9	9.3±0.7	9.8±0.6	1.6±0.1
	+	-	+	-4.0±0.1	-15.0±0.0	8.0±0.6	12.6±1.2	12.2±1.7	15.3±0.4	0.9±0.6
	-	+	+	-	-15.4±0.0	8.4±0.2	-0.1±1.8	11.2±0.3	3.6±0.0	0.2±0.1
	-	+	-	-	-	-	-4.5±1.6	5.2±1.6	6.4±1.6	0.2±0.1

[a)] The difference of metabolite concentrations after 7 days to the initial concentrations. Positive numbers indicate the production of metabolites; negative numbers indicate the consumption of the metabolites.
1, 2-POH = 1,2 propanediol

Table 2. 3. Change of metabolite concentrations after fermentation by *L. diolivorans* in mMRS for 5 days at 30 °C.

	Substrates [mM]			**Metabolites [mM][a)]**								
	Maltose [5 mM]	**1,2-POH [30 mM]**	**Fructose [15 mM]**	**Maltose**	**Fructose**	**Mannitol**	**Lactate**	**Acetate**	**Ethanol**	**1,2-POH**	**Propionate**	**1-POH**
Δ C[a)]	+	+	+	-4.6±0.2	-15.1±0.0	2.6±0.2	16.8±0.2	18.3±0.2	5.9±0.6	-34.1±0.0	7.0±0.1	19.9±2.9
	+	+	-	-4.4±0.2	-	-	1.8±0.5	15.5±0.1	1.2±0.1	-35.8±0.0	8.3±0.2	20.1±2.8
	+	-	+	-4.4±0.2	-14.9±0.0	4.9±0.4	16.0±0.3	12.9±0.3	9.9±1.0	0.0±0.0	2±0.9	0.1±0.0
	-	+	+	-0.3±0.1	-16.5±0.0	1.8±0.3	4.4±1.2	19.6±1.6	1.3±0.1	-37.5±0.0	7.7±0.1	18.2±1.3
	-	+	-	-0.5±0.1	-	-	0.8±1.0	2.7±0.1	0.7±0.1	-37.8±0.0	19.2±1.8	11.3±1.1

[a)] The difference of metabolite concentrations after 5 days to the initial concentrations. Positive numbers indicate the production of metabolites; negative numbers indicate the consumption of the metabolites.
1, 2-POH = 1, 2 propanediol; 1-POH = 1-propanol

2.3.2. Kinetic analysis of *L. buchneri* and *L. diolivorans* cofermentation in sourdough.

L. buchneri and *L. diolivorans* grew to high cell counts in all three sourdoughs prepared with flours of different ash content within two days of incubation and acidified the sourdoughs to a pH of 3.6 (Figure 2.2). In sourdough prepared with Type 550 and Type 1050 flours, cell counts of *L. buchneri* decreased slightly between day three and 14 whereas cell counts of *L. diolivorans* dropped to levels below 10^5 cfu mL^{-1} after 14 days. In wheat 550 sourdough, propionate was detectable after day five. Between day five and 14, levels of acetate increased by around 35 mM. In contrast, amounts of lactate and 1-propanol both decreased by about 5 mM. In wheat 1050 sourdough, propionate was detectable by day two and increased to 48 mM by day 14. At day five, levels of acetate started to increase, whereas lactate and 1-propanol decreased.

In wheat whole grain flour, cell counts of both *L. buchneri* and *L. diolivorans* remained above 10^8 cfu kg^{-1} throughout 14 days of fermentation. Lactate concentrations were higher than in the other sourdoughs (approx. 170 mM). In contrast, the amount of propionate was lowest (12 mM) compared to the other two flours.

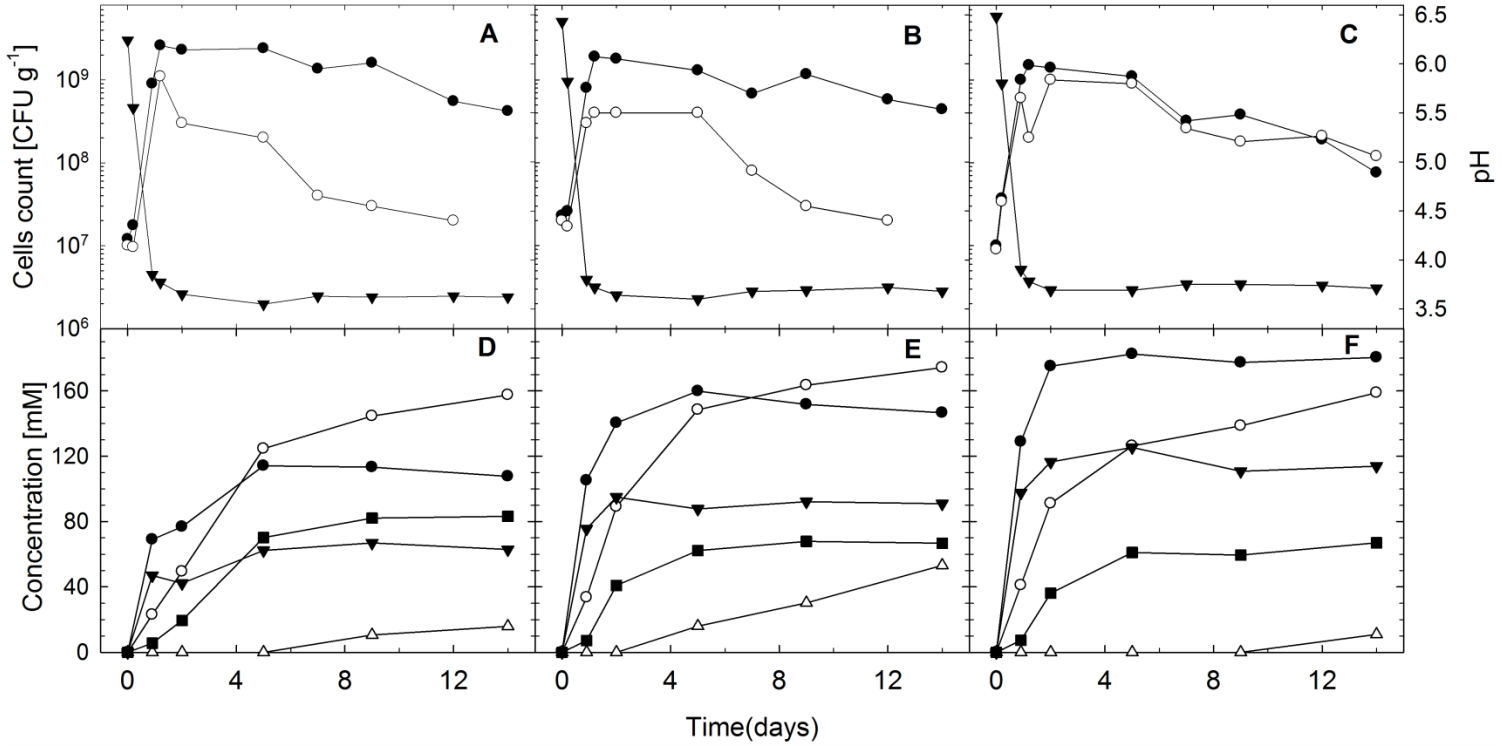

Figure 2.2. Kinetic measurement of dough pH, cells counts and main metabolites during sourdough fermentation with wheat flour Type 550 (low ash content, panels A, D), wheat flour Type 1050 (medium ash content, panel B, E) and whole wheat flour (high ash content, panels C, F) for 14 days at 30 °C. Panels A, B and C show cells counts of *L. buchneri* (●) and *L. diolivorans* (○) and dough pH (∇) during fermentation of sourdough. Panels D, E and F indicate concentrations of lactic acid (●), acetic acid (○), ethanol (∇), propionic acid (Δ), and 1-propanol (■) produced during fermentation.

2.3.3. Antifungal activity.

Baking experiments were performed to evaluate the effect of propionate and acetate formation by *L. buchneri* and *L. diolivorans* on mold growth on sourdough bread. Sourdough prepared from wheat flour Type 1050 was used for the baking experiments because the use of flour with medium ash content resulted in the highest propionate concentrations (Figure 2.2). The addition of 10% sourdough fermented with *L. buchneri* and *L. diolivorans* did not inhibit *P. roquefortii* but deferred the growth of *A. clavatus*, *Cladosporium* spp., and *Mortierella* spp. by one day compared to the control (Table 2.4). The addition of 20% *L. buchneri* and *L. diolivorans* sourdough inhibited growth of *Saccharomyces cerevisiae* at the dough stage (data not shown), inhibited growth on bread of *A. clavatus*, *Cladosporium* spp., and *Mortierella* spp. for more than 12 days and deferred growth of *P. roquefortii* by two days compared to the control. The addition of acetate to commercial BRS sourdough bread deferred the growth of *Cladosporium* spp. by one day; *A. clavatus*, *Mortierella* spp. and *P. roquefortii* were not inhibited. Bread prepared without sourdough but with the addition of propionate deferred growth of *A. clavatus* by three days compared to the control. Growth of *Cladosporium* spp. and *Mortierella* spp. was deferred by one and five days respectively, and *P. roquefortii* was not affected.

Table 2. 4. Time in days to visible growth of molds on sourdough bread stored for 12 days.

	A. clavatus	***Cladosporium* sp.**	***Mortierella* sp.**	***P. roquefortii***
control	3[a)]	3	6	6
5% SD[b)]	3	3	6	6
10%SD	4	4	6	6
20% SD	> 12	> 12	> 12	8
10% BRS[b)]	3	3	6	6
10% BRS-Ac.	3	4	6	6
Propionate	6	4	12	6

[a)] Bread was cut into 1.5 cm wide slices and conidiospores were applied on ten spots per slice to transfer about $4.8*10^3$, $8*10^2$, $8*10^2$, $8*10^1$ conidiospores of *A. clavatus*, *Cladosporium* sp, *Mortierella* sp, and *P. roquefortii* per spot, respectively. Bread slices were wrapped in plastic bags and stored aerobically at room temperature. Mold growth was monitored visually daily for a duration of 12 days. Data correspond to the day when mold growth was visible on all 10 spots.
[b)] SD, experimental sourdough; wheat flour Type 1050 fermented fro 14 days with *L. buchneri* and *L. diolivorans*. BRS, Böcker Reinzucht Sauerteig Rye, fermented for 18 hours at 28 °C; BRS-Ac, BRS sourdough with 0.1% acetate.

2.4. Discussion.

The cofermentation of *L. buchneri* and *L. diolivorans* defers aerobic deterioration of maize silage caused by the growth of aerobic microorganism (Oude Elferink et al, 2001). *L. buchneri* produces acetate and 1-2, propanediol from lactate, which is further metabolized to 1-propanol and propionate by *L. diolivorans* (Holzer et al, 2003). This study provides the characterization of cooperative metabolism of *L. buchneri* and *L. diolivorans* in mMRS and food fermentations, and evaluated the effects of acetate and propionate on mold growth on bread.

The strain of *L. buchneri* used in this study was isolated from an African cereal product (Sekwati-Monang and Gänzle 2007). The cereal isolate *L. buchneri* converted lactate to 1, 2-propanediol in mMRS. Lactate was obtained by the fermentation of maltose or supplied to the growth medium. Higher amounts of 1, 2-propanediol was detected when the strain was grown in the presence of maltose. The alternative metabolites from lactate, acetate and 1, 2-propanediol were not produced in a ratio of 1:1 as reported by Oude Elferink et al (2001), which may be attributable to the more complex carbohydrate composition of the media employed in our study.

The maize silage derived *L. diolivorans* produced the highest amounts of propionate in presence of 1, 2-propanediol as sole carbon source (Krooneman et al, 2002). The ratio of 1-propanol to propionate was close to the ratio of 1.5 to 1 as reported previously if 1, 2 propanediol was the sole substrate (Krooneman et al, 2002). Substantially higher ratios of propanol to propionate were observed in the presence of hexoses as additional substrates.

L. buchneri and *L. diolivorans* grew to high cell counts in wheat, rye and buckwheat sourdoughs (data not shown). In wheat sourdoughs, both strains reached cell counts comparable to sourdough starter cultures growing in wheat, rye, or gluten-free cereals and pseudocereals (Vogelmann et al, 2009). Cell counts of *L. buchneri* were slightly higher in all three sourdoughs. The pH ranged from pH 3.6 to pH 3.7, comparable to levels observed in traditional wheat sourdoughs (Hammes et al, 1996, Gänzle et al, 1998).

Cofermentation of *L. buchneri* and *L. diolivorans* formed lactate, acetate and ethanol in sourdough. Levels of lactate production increased in the order wheat flour type 550, wheat flour type 1050 and whole wheat flour, in keeping with the increased buffer capacities of the flours (Hammes et al, 1996). Remarkably, the levels of acetate were around three to five times higher compared to traditional sourdough inoculated with *L. sanfranciscensis* due to lactate conversion to acetate by *L. buchneri* (Gänzle et al, 1998). During forage preservation by *L. buchneri*, acetate was partially gained from degradation of lactate (Weinberg and Muck 1996).

Independent of the type of flour used, a decrease of lactate content during fermentation correlated to an increase in propionate. Propionate was only detectable after two or more days of fermentation, indicating that propionate was not produced during exponential growth. Formation of 1, 2-propanediol by *L. buchneri* does not support cell growth and requires low pH. The conversion of lactate to propionate was optimal at pH 3.8 compared to pH 4.3 and 5.8. (Oude Elferink et al, 2001).

90% of bread spoilage is caused by *Penicillium* spp.; *Aspergillus, Cladosporium, Mucor, Monilia, Endomyces, Fusarium* and *Rhizopus* spp. have also been identified as bread spoilage fungi (Legan 1993). The use of sourdough delays fungal spoilage due to the formation of organic acids by the lactic acid bacteria starters. Breads containing sourdoughs fermented with various lactobacilli delayed growth of *Aspergillus, Fusarium,* and *Penicillium* spp. (Gerez et al, 2009, Moore et al, 2008, Ryan et al, 2008). Furthermore, the addition of sourdough allowed the reduction of calcium propionate addition (Gerez et al, 2009; Ryan et al, 2008). In the European Union, propionic acids can be added to a maximum level of 0.3%, corresponding to about 40 mM propionate. Up to 48 mM propionate and 175 mM acetate were formed by cofermentation of *L. buchneri* and *L. diolivorans* in wheat flour Type 1050 sourdough. Bread prepared with 20% sourdough accordingly contained about 10 mM propionate and 35 mM acetate. The addition of 20% sourdough almost completely inhibited growth of molds. It is remarkable that 20% addition of experimental sourdough was more effective than either control bread with propionate addition or traditional sourdough bread prepared with additional acetate, which confirms synergistic activity of propionate with other antifungal compounds in sourdough (Ryan et al, 2008).

The addition of 20% experimental sourdough is not suitable for practical applications as high levels of propionate and acetate in bread dough inhibited the activity of baker's yeast (Pattison and von Holy 2001). Preliminary sensory trials indeed indicated strong acidic flavour and taste (data not shown). However, a 10% addition of experimental sourdough, corresponding to 5 and 17 mM

propionate and acetate, respectively, delayed growth of two molds by one day relative to the control breads prepared from straight dough or with traditional sourdough without adverse effects on yeast activity of bread quality. Sourdoughs cofermented with *L. diolivorans* and *L. buchneri* exhibited increased antifungal properties in comparison with other studies on antifungal starter cultures for bread production that reported no difference between experimental and traditional sourdoughs (Dal Bello et al, 2007, Moore et al, 2008) or a one day difference between experimental sourdough with antifungal starter culture and traditional sourdough with *L. sanfranciscensis* (Ryan et al, 2008) when sourdoughs were added at 20%. Elongated delay of mold growth was only achieved when bread was fully fermented with sourdough starter culture, however, this process is not applied in baking practice (Gerez et al, 2009; Lavermicocca et al, 2000).

In conclusion, cofermentation with *L. buchneri* and *L. diolivorans* allows the fermentative accumulation of propionate and acetate in sourdough and represents a process to increase antifungal capacities of bread.

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Chapter 3: Metabolic pathway of α-ketoglutarate in *Lactobacillus sanfranciscensis* and *Lactobacillus reuteri* during sourdough fermentation

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3.1. Introduction.

Sourdoughs harbour stable and symbiotic cultures of yeast and lactobacilli. Sourdough has a long history of use as leavening and acidulant in bread baking but was replaced by straight dough processes and chemical acidulants in the last century. This trend has been reversed in the past years because the use of sourdough improves nutritional properties, flavour, texture, and shelf life of baked goods (Arendt et al, 2007). Amino acid metabolism by lactobacilli contributes to bread quality because metabolites from arginine, leucine, isoleucine, and phenylalanine are active as flavour compounds, precursors for generation of flavour compounds during baking, or antifungal compounds (Lavermicocca et al, 2003, Gänzle et al, 2007).

Transaminases are key enzymes for the conversion of amino acids by lactic acid bacteria (Liu et al, 2008; Smit et al, 2005). Although transaminases of lactobacilli and their contribution to amino acid metabolism during food fermentations are not as well characterized as lactococcal enzymes, evidence for their contribution to amino acid turnover by lactobacilli employed in cheese, sausage, and sourdough fermentations has been provided in the past years (Larrouture et al, 2000; Thage et al, 2004; Vermeulen et al, 2006). Transaminases convert amino acids to the corresponding α-keto acid, in turn, another keto acid acts amino group acceptor and is converted to the corresponding amino acid. The preferred amino acid acceptor in lactic acid bacteria is α-KG and the corresponding product is glutamate (Tanous et al, 2005; Engels et al, 2000). Pyruvate functions as alternative amino group acceptor, however, the efficiency of α-KG is much higher

than pyruvate (Yvon et al, 2000). Amino acid metabolism through transamination is particularly relevant during ripening of dry fermented meats and cheese because microbial metabolites generated from amino acids are main contributors to the flavour (Berdagué et al, 1993; Yvon et al, 1997); however, amino acid metabolism by staphylococcus has a stronger impact to flavour development in fermented meats compared to lactobacilli (Larrouture et al, 2000). Addition of α-KG to cheese and meat fermentations enhanced formation of flavour volatiles through the transaminase pathway (Yvon et al, 1998; Casey et al, 2004; Tjener et al, 2004).

Different from meat and dairy fermentations, obligate hetero-fermentative lactobacilli generally prevail in sourdough fermentations and the species *L. sanfranciscensis* and *L. reuteri* are considered key organisms in type I and type II sourdoughs, respectively (Vogel et al, 1999; Gänzle et al, 2007). In contrast to *Lactobacillus plantarum* and other homo-fermentative or facultative hetero-fermentative lactobacilli, addition of α-KG did not enhance transamination of phenylalanine by *L. sanfranciscensis* (Vermeulen et al, 2006), which implied the existence of other pathway for α-KG metabolism. Moreover, use of α-KG as amino group acceptor in the transaminase reaction was observed only if citrate or fructose were present as alternative electron acceptors (Vermeulen et al, 2006).

Evidence that α-KG is employed as electron acceptor by strains in the genera *Lactobacillus, Pediococcus, Leuconostoe* and *Streptococcus* and converted to 2-hydroxyglutarate (2-OHG) was previously provided by Radler and Bröhl (1984). A majority of strains of lactobacilli, 72 out of 82, metabolized α-KG to 2-OHG.

However, the strains used were not classified on species level and metabolites from α-KG were not quantified.

This study aimed to identify metabolic pathways of α-KG and its alternative fate as electron- or amino group acceptor in two sourdough isolates. *L. sanfranciscensis* DSM 20451^{T} and *L. reuteri* LTH 2584 are isolates from type I and type II sourdoughs, respectively (Kline and Sugihara, 1971; Gänzle and Vogel, 2002; Böcker et al, 1995). Experiments were carried out in laboratory media and in model wheat sourdough fermentations.

3.2. Materials and methods.

3.2.1. Strains, media and culture conditions.

The sourdough isolates *L. sanfrancisencis* DSM 20451^T (Kline and Sugihara, 1971) and *L. reuteri* LTH 2584 (Böcker et al, 1995) were grown in mMRS medium containing 0.5% beef extract, 0.5% yeast extract, 1% peptone, 0.3% NH_4Cl, 0.4% K_2HPO_4, 0.26% KH_2PO_4, 0.01% $MgSO_4$ x $7H_2O$, 0.005% $MnSO_4$ x $4H_2O$, 0.1% Tween 80, 0.05% L-cysteine-HCl, and 0.0002% each of vitamins B1, B2, B6, B5, B12, and B9. Maltose, α-KG, citrate, and phenylalanine were added after autoclaving as described in the following sections. The pH of the media was adjusted to 6.2 and *L. sanfrancisencis* and *L. reuteri* were incubated at 30 and 37 °C, respectively.

3.2.2. Analysis of metabolites with HPLC.

Organic acids, alcohols, and sugars were determined with HPLC (Agilent 1200 series, Agilent, Santa Clara, USA) equipped with an Aminex HPX-87 column (300 mm x 7.8 mm, Biorad, Mississauga, Canada) and was eluted with 0.4 mL min^{-1} with 5 mM H_2SO_4 at 80 °C. The injection volume was 10 μL and analytes were quantified by refractive index and UV (210 nm) detection. Concentrations of maltose, lactate, acetate, ethanol, α-KG, 2-OHG, γ-hydroxybutyrate, succinate, and phenyllactate were determined using external standards. Prior to analysis, samples were mixed with 70% perchloric acid to a concentration of 3.7%, incubated at 4 ˚C overnight, and precipitates were removed by centrifugation.

Concentrations of amino acids and γ-aminobutyrate (GABA) were analyzed after derivatization with o-phthaldialdehyde (OPA) as previously described (Sedgwick et al, 1991). OPA derivatives were separated using Varian 5000 series HPLC (Varian, Palo Alto, California, USA) equipped with a Supelcosil 3 µm LC-18 column (150 x 4.6 mm, Sigma, Germany) at a flow rate of 1.1 mL min^{-1} with 0.1 M sodium acetate buffer and methanol as the eluents. The injection volume was 10 µL and samples were detected by Varian fluorichrom detector with exitation wavelength of 340 nm and emission wavelength of 450 nm.

3.2.3. Analysis of metabolites with GC.

Quantification of 2-OHG was performed with GC on a Varian 3400 series instrument (Varian, Palo Alto, California, USA) equipped with a Stabilwax DA column (30 m x 0.53 mm, 0.5 µm film thickness, Restek, Bellefonte, USA) and a flame ionization detector. Helium was used as carrier gas at 7.5 psi head pressure. The column oven was set to 80 °C and the temperature was increased by 10 °C min^{-1} to 170 °C and held for 10 minutes. The injection port was set to 170 °C and the detector was set at 190 °C. Samples were prepared by adding phosphoric acid to a final concentration of 4% into standard solutions, culture supernatants, or fermentation liquor from sourdough. The syringe was washed with methanol after injection. The assay was calibrated with external standards containing 0.1, 0.2, 0.5, 1, and 2 mM 2-OHG and the calibration curve had an r^2 of 0.9918.

3.2.4. Identification of metabolites produced from conversion of α-KG by *L. sanfrancisencis* and *L. reuteri*.

To identify and to quantify metabolites produced by *L. sanfrancisencis* and *L. reuteri* in presence of maltose and α-KG, overnight cultures were washed once in mMRS and 1 mL mMRS was inoculated with 5 µL cell suspension. The concentration of maltose and α-KG were adjusted by adding filter-sterilized stock solutions of the substrates or water to the autoclaved media. The maltose concentration was adjusted to 13 mM or 23 mM as indicated, and the α-KG concentration was 0 or 10 mM. Cultures were incubated for 48 hours and concentrations of maltose, α-KG, lactate, acetate, ethanol and amino acids were determined by HPLC as described above. To verify 2-OHG formation by *L. sanfrancisencis* and *L. reuteri*, culture supernatants were additionally analyzed by GC as described above. Results are presented as means ± standard deviations of duplicate experiments.

3.2.5. Determination of OHG dehydrogenase activity in *L. sanfranciscensis* and *L. reuteri.*

Crude cell extracts were prepared from 5 mL overnight culture of *L. sanfrancisencis* and *L. reuteri* in mMRS containing 23 mM maltose or 23 mM maltose and 10 mM α-KG as substrates. Cells were harvested by centrifugation, washed with 1 mL reaction buffer (RB) containing 25 mM citrate, 25mM imidazole, and 25 mM KH_2PO_4, pH 4.4, and resuspended in the same buffer. Cells were mixed with 0.7 mL of 0.1 mm glass beads (Biospec products, Inc,

Bartlesville, USA) and disrupted with bead beater (Mini-beadbeater-8**,** Biospec products, Inc, Bartlesville, USA) for 3 cycles and 1 minute per cycle. Samples were cooled on ice between cycles. Cell debris was removed by centrifugation at 8400 rcf for 5 minutes and the supernatant was immediately used for enzymatic test. Enzymatic reactions were performed in buffer containing 25 mM imidazole, 25 mM KH_2PO_4, 5 mM α-KG, 0.1 mM NADH, pH 5.4 for *L. sanfranciscensis* and 25 mM citrate, 25 mM imidazole, 25 mM KH_2PO_4, 5 mM α-KG, 0.1 mM NADH, pH 4.4 for *L. reuteri*, respectively. The enzymatic reaction was initialized by adding 0.1% crude cells extract and samples were incubated at 30 °C and 37 °C for *L. sanfranciscensis* and *L. reuteri,* respectively. NADH was quantified by measuring the absorbance at 340 nm by UV spectrophotometer every 3 minutes for 1 hour. The protein concentrations of crude cells extracts were measured by the Bradford method (Bio-Rad, USA) using bovine serum albumin as standard. The enzyme activity expressed as mM NADH (min x mg protein)$^{-1}$, and results were presented as means ± standard deviations of two independent experiments.

3.2.6. Influence of α-KG, citrate, and phenylalanine on kinetics of growth and metabolite formation of *L. sanfranciscensis* and *L. reuteri*.

Overnight cultures of *L. sanfrancisencis* and *L. reuteri* were washed with mMRS, resuspended in the same volume of fresh medium, and 40 µL cell suspension was used to inoculate 5 mL of mMRS. Media contained 23 mM maltose, 0 or 10 mM α-KG, 0 or 10 mM citrate, and 0 or 10 mM phenylalanine. The cultures were incubated for 48 hours and samples were taken after 0, 2, 4, 8, 16, 24, or 48 hours.

The cell density in samples was determined by measuring the optical density at 600 nm. Concentrations of maltose, lactate, acetate, ethanol, α-KG and phenyllactate were analyzed by HPLC as described. Fermentations were conducted in duplicate and results are presented as means ± standard deviations.

3.2.7. Influence of α-KG on metabolite formation by *L. sanfrancisencis* and *L. reuteri* during growth in sourdough.

Overnight cultures of *L. sanfrancisencis* and *L. reuteri* in mMRS containing 23 mM maltose and 11.5 mM fructose were washed with an equal volume of tap water containing 23 mM maltose and 0 or 40 mM α-KG, and resuspended in the same solution. Ten mL of tap water containing the inoculum, maltose and α-KG were used for dough preparation with 10g white wheat flour obtained at a local supermarket. Doughs were incubated at 30 or 37°C and samples were taken after 0, 2, 4, 8, 16, 24, or 48 hours of fermentation. Sourdough was diluted five fold and analysed by HPLC as described before to quantify maltose, lactate, acetate, ethanol, and α-KG. Production of 2-OHG was quantified by GC. Fermentations were conducted in duplicate and results were presented as means ± standard deviations.

3.3. Results.

3.3.1 Identification of metabolites produced from conversion of α-KG by *L. sanfrancisencis* and *L. reuteri*

Metabolites produced from utilization of maltose and α-KG were quantified to determine whether α-KG was used as an electron acceptor. Metabolite concentrations after 48 hours are shown in Table 3.1. Both *L. sanfranciscensis* and *L. reuteri* produced around 4 mM more acetate in presence of maltose and α-KG compared to the presence of maltose only, equivalent to the utilization of 8 mM electron acceptor to regenerate NADH. Around 2 mM and 6 mM less ethanol were obtained in the corresponding samples for *L. sanfranciscensis* and *L. reuteri*, respectively.

HPLC analysis of cultures supernatants did not show the presence of α-hydroxybutyrate or succinate, which were previously described as metabolites of α-KG in lactic acid bacteria (Radler and Bröhl, 1984). Moreover, amino acid analysis revealed no significant differences in the amino acids concentrations, including concentrations of glutamine, glutamate, or GABA, after fermentation in mMRS with or without α-KG by *L. sanfranciscensis* and *L. reuteri* (data not shown). However, an additional peak was detected by HPLC in both *L. sanfranciscensis* and *L. reuteri* in presence of maltose and α-KG compared to the sample fermented with maltose only (data not shown). External 2-OHG standard showed the similar retention time but peak resolution with HPLC method was poor (data not shown). GC analysis confirmed that 2-OHG was produced from conversion of α-KG by both *L. sanfranciscensis* and *L. reuteri* (Figure 3.1).

Table 3. 1. Change in concentration of metabolites during fermentation in mMRS with additional 13 mM maltose and/or 10 mM α-KG for 48 hours at pH 6.2 and 30°C by *L. sanfranciscensis* and pH 6.2 and 37 °C by *L. reuteri*, respectively.

	α-KG	**Maltose mM**	**α-KG mM**	**Lactate mM**	**Acetate mM**	**Ethanol mM**
L. sanfranciscensis	+	-11.6±0.1	-11.2±0.0	28.9±0.4	6.5±0.3	26.1±0.2
	-	-10.6±0.1	-	23.5±0.5	2.2±0.8	28.1±0.5
L. reuteri	+	-13.4±0.0	-11.2±0.0	31.3±0.3	4.8±0.1	31.7±1.3
	-	-13.2±0.0	-	29.5±0.0	1.7±0.2	37.1±0.2

Positive numbers indicate the production of metabolites; negative numbers indicate the consumption of the metabolites.

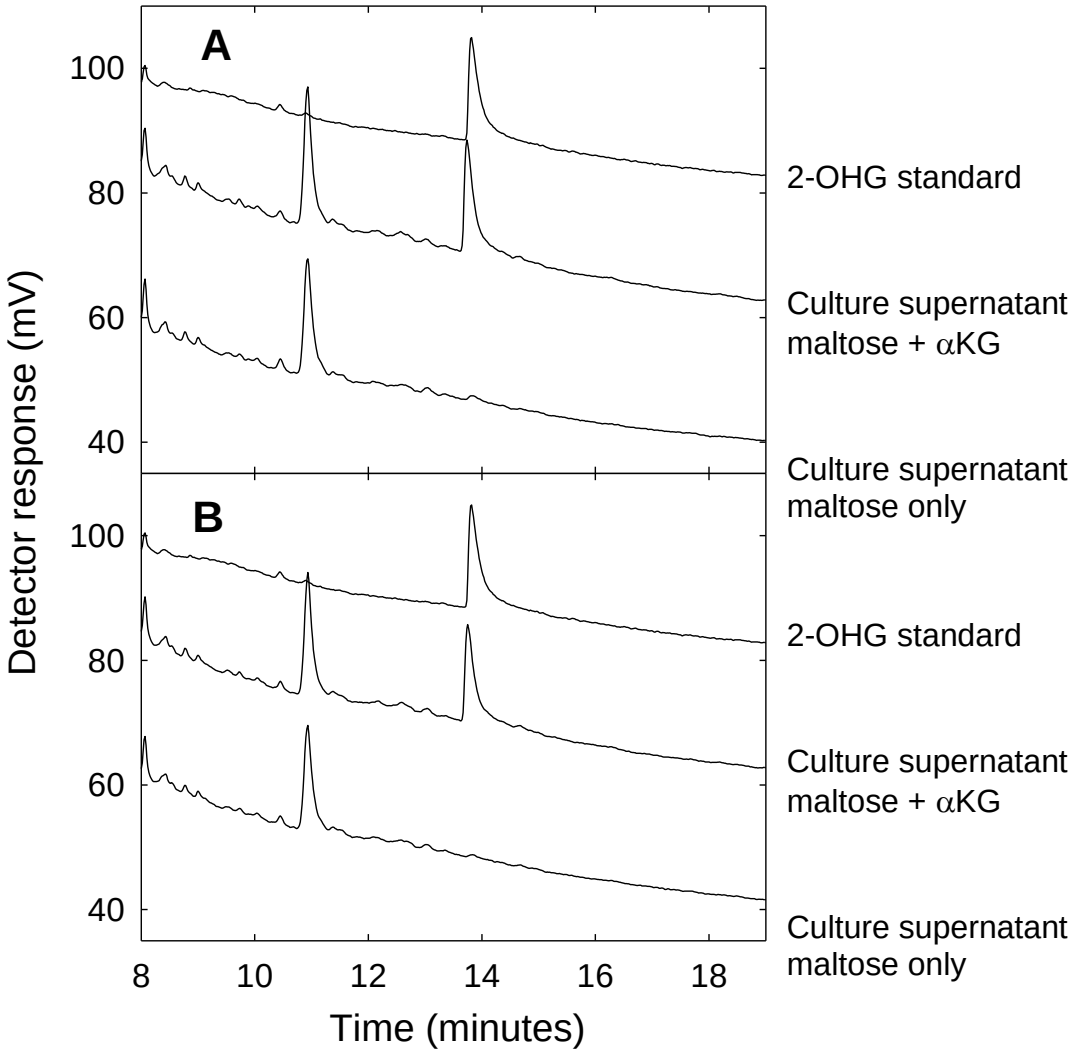

Figure 3.1. Gas chromatographic quantification of 2-OHG produced by *L. sanfranciscensis* (Panel A) and by *L. reuteri* (Panel B) in mMRS media containing maltose and α-KG as substrates. Upper traces, injection of 4 mM L^{-1} 2-OHG standard; middle traces, culture supernatant of mMRS containing 23 mM maltose as carbon source and 10 mM α-KG as electron- or amino group acceptor; lower traces, culture supernatant of mMRS containing 23 mM maltose.

3.3.2. Determination of OHG dehydrogenase activity in *L. sanfranciscensis* and *L. reuteri*.

To verify that α-KG was converted to 2-OHG in a NADH dependent reaction, conversion of NADH to NAD^+ by crude cell extract was measured in presence of α-KG. Crude cells extract of *L. sanfranciscensis* grown with or without additional α-KG had enzymatic activity of 6.25±0.31 and 2.46±0.86 mM NADH (min x mg protein)$^{-1}$ whereas the corresponding enzymatic activity of *L. reuteri* was 1.20±0.19 and 1.11±0.05 mM NADH (min x mg protein)$^{-1}$, respectively.

3.3.3. Influence of α-KG, citrate, and phenylalanine on kinetic growth and metabolite formation of *L. sanfranciscensis* and *L. reuteri.*

Growth of *L. sanfranciscensis* and *L. reuteri* with maltose as carbon source is generally accelerated when fructose or other electron acceptors are present (Stolz et al, 1995; Gänzle et al, 2007). The determination of the growth kinetics thus enables a first assessment whether a substrate is used as alternative electron acceptor by *L. sanfranciscensis* or *L. reuteri*. Addition of α-KG to mMRS-maltose highly accelerated growth of *L. sanfranciscensis* (Figure 3.2). Culture grown in presence of α-KG reached an OD_{600nm} of 3 within 24 hours. A comparable growth was reached only after 48 hours when maltose was the sole substrate. Citrate or phenylalanine did not influence growth (Figure 3.2). Growth of *L. reuteri* was highly accelerated in presence of α-KG compared to the sample fermented with maltose and / or citrate. Fast growth was observed in the sample with maltose, α-KG and in presence or absence of citrate. Citrate also stimulated growth.

Remarkably, phenylalanine deferred growth of *L. reuteri* when citrate was also present.

Consumption of maltose (data not shown) by *L. sanfranciscensis* and production of lactate, acetate and ethanol was highly accelerated in presence of α-KG compared to cultures fermented with maltose only, or maltose and citrate (Figure 3.3A, 3.3C and 3.3E). Alpha-KG was quantitatively consumed within the first hours of growth (data not shown). Acetate levels were about 4 mM higher in presence of maltose and α-KG and 7 mM higher in presence of maltose, α-KG, and citrate compared to cultures fermented with maltose only, in keeping with 2-OHG levels of 3.6±1.1 and 3.4±0.2 after 48 hours of incubation, and partial utilization of citrate (Figure 3.3C). Addition of phenylalanine and citrate redirected the role of α-KG from electron acceptor to amino group acceptor. Acetate and 2–OHG concentrations were 9 mM and 3.9 ± 0.3 mM, respectively, when maltose, α-KG, and phenylalanine were offered as substrate but were reduced to 6 mM acetate and 2.1±0.4 mM 2-OHG when citrate was present in addition to maltose, α-KG and phenylalanine.

In *L. reuteri,* addition of α-KG accelerated maltose utilization (data not shown) and lactate production compared to cultures containing maltose only or maltose and citrate (Figure 3.3B). Lactate and ethanol production were deferred when phenylalanine was present. Acetate levels ranged from 4 – 6 mM in presence of α-KG and from 1 – 2 mM in absence of α-KG. Citrate levels remained unchanged during 48 hours of fermentation (data not shown) and citrate addition hence did not alter acetate and ethanol formation (Figure 3.3D and 3.3F). Similar

concentrations of ethanol were observed after 24 hours in all samples. Increased acetate levels in presence of α-KG corresponded to accumulation of 3.2±0.4 and 3.2±0.2 mM 2-OHG in presence of maltose or maltose and citrate, respectively. 2-OHG levels were 3.4±0.0 mM when phenylalanine was present in addition to maltose and α-KG; however, only 2.5±0.1 mM 2-OHG after growth of *L. reuteri* in media containing maltose, α-KG, citrate, and phenylalanine. Comparable to *L. sanfranciscensis*, this result indicates that addition of phenylalanine and citrate partially redirected the use of α-KG from electron acceptor to amino group acceptor.

L. sanfranciscensis produced only low levels of phenyllactate, 17±2 and 14±1 μM, in presence of maltose or maltose and citrate as carbon sources, respectively. Alternative metabolites of phenylalanine, i.e. phenylethanol, phenylacetaldehyde, and phenylacetate were not detected (data not shown). Phenyllactate levels increased to 36±3 and 51±2 μM when α-KG added to media containing maltose or maltose and citrate, respectively. Addition of phenylalanine and α-KG increased phenyllactate formation by *L. sanfranciscensis* to 80±2 and 96±0 μM after fermentation of media with maltose or maltose and citrate, respectively. *L. reuteri* accumulated 44±1 μM phenyllactate in media containing maltose. 54±6, 55±1, and 57±1 μM phenyllactate were produced in media containing maltose and α-KG, maltose and citrate, or maltose with α-KG and citrate, respectively. Addition of phenylalanine increased phenyllactate levels to 138±2, 161±17 μM in media containing maltose and α-KG or maltose, α-KG, and citrate as co-substrates, respectively. Although only a small fraction of phenylalanine was

converted to phenyllactate, these results support the interpretation that α-KG was preferentially used as electron acceptor by *L. sanfranciscensis* and *L. reuteri* unless citrate redirected the use of α-KG from electron acceptor to amino group acceptor.

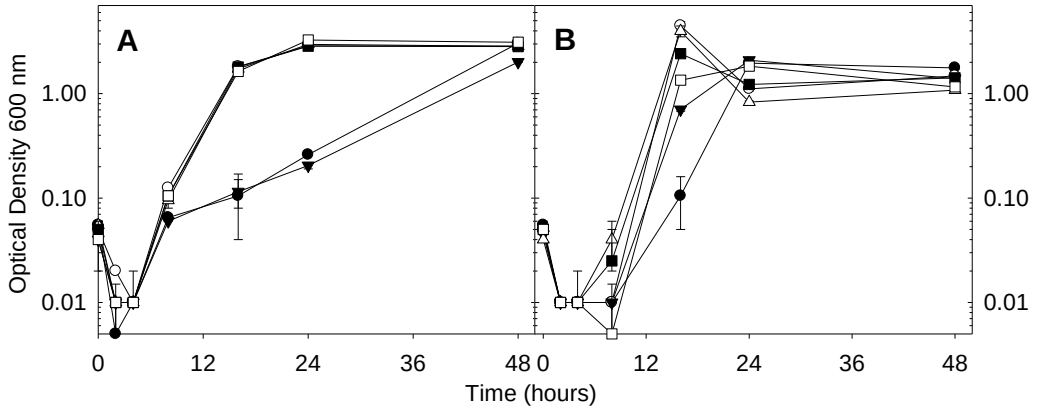

Figure 3.2. Growth of *L. sanfranciscensis* (Panel A) and *L. reuteri* (Panel B) in mMRS media. All media contained 20 mM maltose as carbon source and the following additional substrates: 10 mM α-KG as electron- or amino group acceptor, 10 mM citrate as alternative electron acceptor, and 10 mM phenylalanine as amino group donor. Symbols indicate growth in media containing maltose only (●), maltose and α-KG (❍), maltose and citrate (♦), maltose, α-KG, and citrate (Δ), maltose, α-KG, and phenylalanine (■), or maltose α-KG, citrate, and phenylalanine (□). Results are presented as means ± standard deviations of two independent experiments.

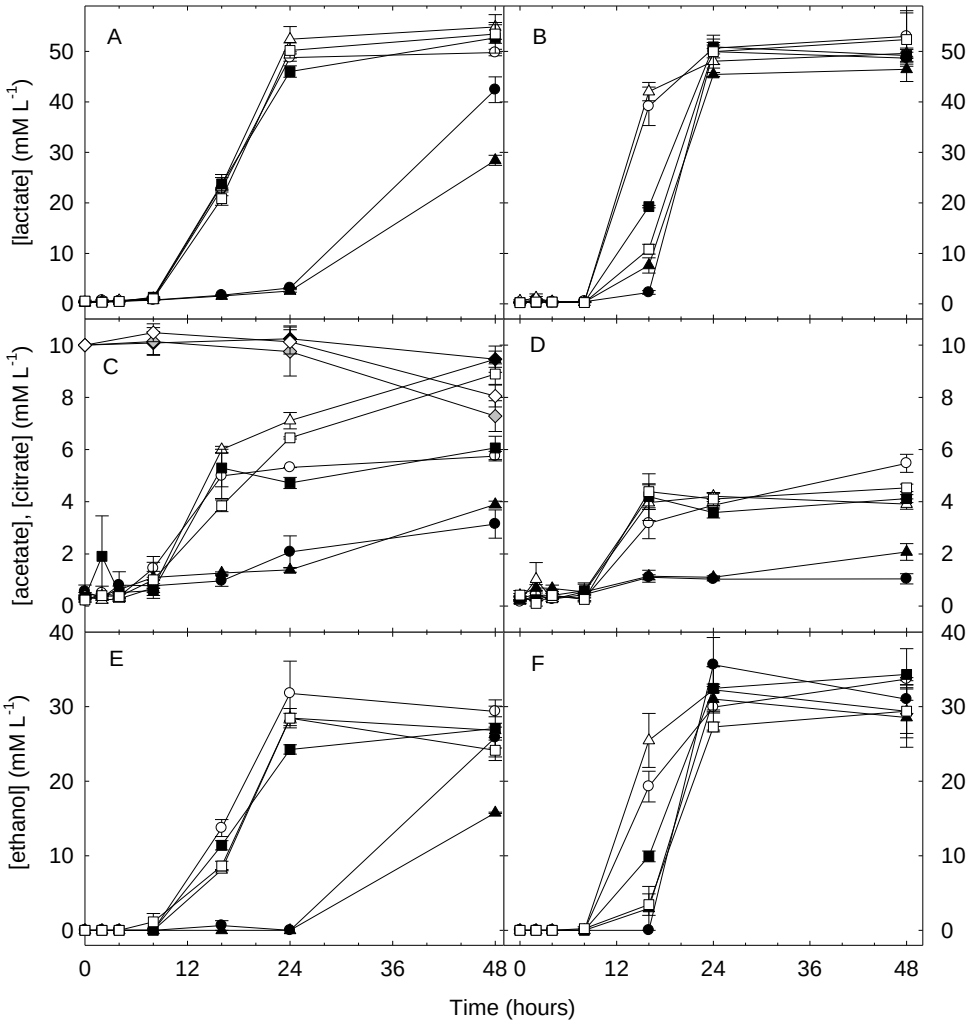

Figure 3.3. Kinetic of metabolite formation by *L. sanfranciscensis* (Panels A, C, and E) and *L. reuteri* (Panel B, D, and F) in mMRS media. All media contained 20 mM maltose as carbon source and the following additional substrates: 10 mM α-KG as electron- or amino group acceptor, 10 mM citrate as alternative electron acceptor, and 10 mM phenylalanine as amino group donor. Panels indicate lactate concentrations (panels A and B), acetate concentrations (Panels C and D), and ethanol concentrations (panels E and F) and symbols indicate metabolite formation in media containing maltose only (●), maltose and α-KG (❍), maltose and citrate (♦), maltose, α-KG, and citrate (Δ), maltose, α-KG, and phenylalanine (■), or maltose, α-KG, citrate, and phenylalanine (□). Panel C additionally depicts citrate concentrations in media containing maltose and citrate (◆), maltose, α-KG, and citrate (◆), or maltose, α-KG, citrate, and phenylalanine (◇). Results are presented as means ± standard deviations of two independent experiments.

3.3.4. Influence of α-KG on metabolite formation by *L. sanfrancisencis* and *L. reuteri* during growth in sourdough.

To confirm conversion of α-KG to 2-OHG in wheat sourdough fermentation, wheat sourdoughs were fermented in presence and absence of 20 mM α-KG and metabolites quantified during 48 hours of fermentation (Figure 3.4). Similar amounts of maltose were detected throughout the fermentation in *L. sanfranciscensis*. Concentrations of lactate and acetate were slightly higher in presence of α-KG compared to the control. Likewise, addition of α-KG did not affect maltose levels in sourdoughs fermented with *L. reuteri* but supported production of slightly higher concentrations of lactate and acetate. Concentrations of 2-OHG in sourdoughs with addition of α-KG were 11.8±2.5 and 11.2±1.1 mM after fermentation by *L. sanfranciscensis* and *L. reuteri*, respectively.

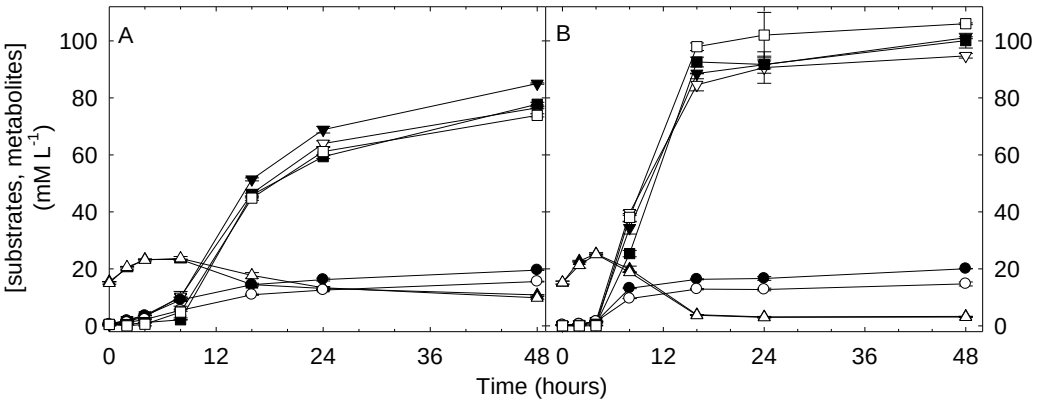

Figure 3.4. Kinetic of metabolite formation by *L. sanfranciscensis* (Panel A) and *L. reuteri* (Panel B) during growth in sourdough. Sourdoughs were prepared with wheat flour and water (open symbols) or with wheat flour, water, and addition of 20 mM αKG (closed symbols). The following substrates and metabolites are depicted: maltose (♦ , Δ), acetate (●, ❍), ethanol (■, ❑), and lactate (⧫, ◊). Results are presented as means ± standard deviations of two independent experiments.

3.4. Discussion.

Pathways for α-KG metabolism in lactobacilli include its use as amino group acceptor in the transaminase reaction, and NADH-dependent reduction to 2-OHG (Figure 3.5). This study presents the first quantitative analysis of the alternative products from α-KG in lactic acid bacteria, and demonstrated that α-KG was used preferably as electron acceptor by the obligate heterofermentative *L. sanfranciscensis* and *L. reuteri*. Moreover, the presence of citrate and amino acids determined its fate as electron acceptor or amino group acceptor.

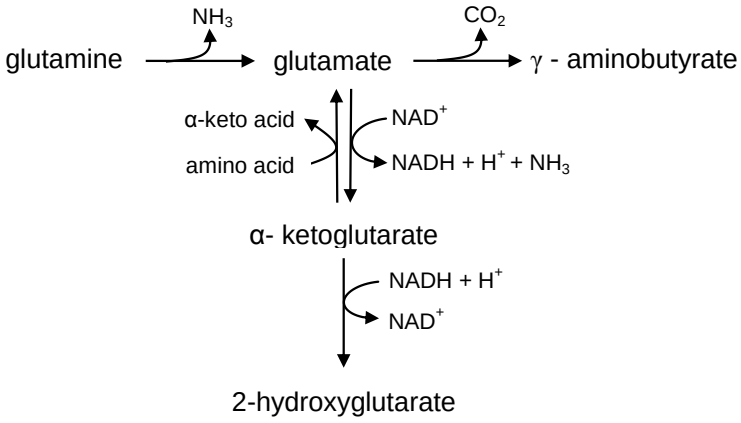

Figure 3.5. Overview on glutamine metabolism in lactobacilli Drawing according to Radler and Bröhl 1984; Ueno et al, 1997; Weingand-Ziadé et al, 2003; Tanous et al, 2005.

OHG was quantified directly by GC after acidification with phosphoric acid. Previous GC protocols to quantify the non-volatile lactate and OHG were based on extraction and derivatisation (Borenstein et al, 1982; Jokipii et al, 1987, Gibson 1993; Struys et al, 2003). The sensitivity of the GC method sufficed for the detection of millimolar amounts of 2-OHG. Determination of phenyllactate was based on the HPLC method described by Vermeulen et al, (2006) to allow quantification of maltose, fructose, mannitol, glucose, lactic acid, acetic acid, ethanol, and phenyllactate in a single HPLC run. Although GC methods for detection of phenyllactate offer increased sensitivity and an improved peak resolution (Valerio et al, 2004; Armaforte et al, 2006; Ryan et al, 2009), quantification of phenyllactate produced by *L. plantarum* in wheat sourdough by

HPLC and GC provided comparable results (Vermeulen et al, 2006; Ryan et al, 2009).

Utilization of citrate as in both *L. sanfrancicensis* and *L. reuteri* is strain dependent (Radler and Bröhl 1984; Kaneuchi et al, 1988; Stolz et al, 1995a, 1995b; Østlie et al, 2003). Strains of *L. sanfranciscensis* and *L. reuteri* that are capable of citrate utilization consumed only a small fraction of the citrate after 50 hours of incubation (Stolz et al, 1995a, 1995b). Citrate utilization in lactic acid bacteria contributes to pH homeostasis and acid tolerance (Konings 2001) and citrate utilization by *L. plantarum* is induced by a low pH (Kennes et al, 1991; Lindgren et al, 1990). In this study, acetate formation from citrate by *L. sanfranciscensis* was observed only during the stationary phase of growth. Remarkably, citrate utilization was stimulated by addition of α-KG in *L. sanfranciscensis*.

In keeping with its use as electron acceptor by *L. sanfranciscensis* and *L. reuteri*, addition of α-KG accelerated their growth in mMRS-maltose, and supported formation of acetate instead of ethanol. Quantification of 2-OHG by GC indicated conversion of 40 – 50% of α-KG to the corresponding hydroxy-acid by *L. sanfranciscensis* and *L. reuteri*. *Oenococcus oenos* produces the alternative products γ-hydroxybutyrate and succinate from α-KG (Kapol et al, 1990; Radler and Bröhl 1984); however, these products were not detected in culture supernatants in this study. Likewise, glutamate or GABA accumulation by *L. sanfranciscensis* or *L. reuteri* was not influenced by the presence of α-KG. NADH-dependent OHG dehydrogenase activity was confirmed by enzymatic

analysis of crude cell extracts of *L. sanfranciscensis* and *L. reuteri.* Genomic analysis demonstrates that *L. reuteri* and *L. brevis* harbour a multitude of α–hydoxy acid dehydrogenases but their substrate specificity remains unknown (Liu et al, 2008). In *Escherichia coli*, 3-phosphoglycerate dehydrogenase catalyzes the conversion of α-KG to 2-OHG (Zhao and Winkler, 1996).

Homo-fermentative lactic acid bacteria such as *Lactococcus lactis* (Yvon et al, 1997; Banks et al, 2001; Engels et al, 2000), *Lactobacillus delbrueckii* subsp. *lactis, Lactobacillus helveticus,* and *Streptococcus thermophilus* (Helinck et al, 2004) utilize α-KG preferentially as amino group acceptor in the transaminase reaction. In *Lactococcus lactis,* the transamination of aromatic and branched-chain amino acids by aromatic aminotransferase AraT (Yvon et al, 1997; Gao and Steele 1998), and transamination of leucine and isoleucine by the branched-chain aminotransferase BcaT (Yvon et al, 2000) is stimulated by α-KG addition. However, conversion of phenylalanine to phenyllactate was increased in *L. plantarum* but not in *L. sanfrancicensis* when α-KG was added as amino-group acceptor in sourdough (Vermeulen et al, 2006).

L. sanfranciscensis and *L. reuteri* produced 15 – 50 µM phenyllactate in mMRS, the concentrations increased to 80 – 160 µM when an excess of phenylalanine was added to the medium. These concentrations are generally in agreement with phenyllactate levels previously reported for strains of *L. sanfranciscensis* and *L. plantarum* during growth in nitrogen limited medium (20 - 80 µM, Vermeulen et al, 2006), mMRS (200 – 350 µM, Valerio et al, 2004) and sourdough (100 – 400 µM, Vermeulen et al, 2006; Ryan et al, 2009). In keeping with prior observations

(Vermeulen et al, 2006), α-KG addition to mMRS-maltose had only minor effects on phenyllactate production in *L. sanfranciscensis* and *L. reuteri*. Remarkably, the use of α-KG as amino group acceptor in *L. sanfranciscensis* during growth in nitrogen-limited mMRS and in sourdough is dependent on the addition of alternative electron acceptors such as citrate or fructose (Vermeulen et al, 2006). NADH consumption by alternative electron acceptors will increase the availability of oxidized cofactors for the α-KG forming glutamate dehydrogenase reaction, and decrease the availability of reduced cofactors for the α-KG consuming 2-OHG dehydrogenase (Figure 3.5) (Vermeulen et al, 2006).

The formation of alternative amino group acceptors in citrate metabolism additionally contributes to its effect on phenylalanine metabolism. Stimulatory effects of citrate metabolism on phenylalanine transamination in *L. lactis* were attributed to the accumulation of pyruvate and oxaloacetate as alternative amino group acceptors; formation of α-KG from citrate through aconitase and isocitrate dehydrogenase activity was ruled out (Tanous et al, 2005). The present study provides evidence that addition of citrate reduced conversion of α-KG by NADH-dependent dehydrogenases to 2-OHG in *L. sanfranciscensis* and *L. reuteri*, and thus increased its availability as amino group acceptor. The concentration of 2-OHG were lowest in cultures of *L. sanfranciscensis* and *L. reuteri* when citrate and phenylalanine were present in addition to α-KG, and low 2-OHG yields corresponded to high phenyllactate concentrations. In *Lactococcus lactis*, however, citrate is capable of stimulating phenylalanine conversion in the absence of glutamate or α-KG whereas the stimulatory effects in *L. sanfranciscensis* and

L. reuteri were dependent on the simultaneous presence of glutamate or α-KG (Tanous et al, 2005; Vermeulen et al, 2006, this study).

In conclusion, this study demonstrated that the obligate heterofermentative *L. sanfranciscensis* and *L. reuteri* primarily utilised α-KG as electron acceptor. In contrast to homofermentative lactic acid bacteria, the use of α-KG as amino group acceptor occurred only in presence of abundant amino donors, e.g. phenylalanine, and citrate. Addition of α-KG to the fermentation substrate, a strategy that was successfully employed to enhance flavour formation in cheese and sausage fermentation (Yvon *et el.* 1998; Tjener et al, 2004), is therefore not generally suitable in sourdough fermentations. Because the α-KG is used as electron acceptor in heterofermentative lactobacilli, the presence of alternative electron acceptor impacts the metabolic flux in the transamination pathway during sourdough fermentation (Vermeulen et al, 2006). Further studies are required for improved understanding of parameters that control the alternative use of α-KG as electron acceptor or amino group acceptor.

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Chapter 4: Comparative study of acid resistance pathways in *Lactobacillus reuteri* 100-23 during sourdough fermentation

4.1. Introduction.

Based on technology of sourdough production, traditional sourdough can be divided into four types: Types 0, I, II, and III (De Vuyst and Neysens, 2005). Microbiota involved in sourdough fermentation largely depends on the parameters of dough processing. Type 0 sourdough is sponge dough fermented by baker's yeast. Microbiota in Type I sourdoughs is maintained without baker's yeast, with leavening provided instead by continuous back slopping. This sourdough is characterized by its rapid growth at ambient temperature. Dominant *Lactobacillus* strains isolated from Type I sourdough such as *L. sanfranciscensis* are more sensitive to low pH compared to strains from Type II sourdough. Type II sourdough are employed as acidifiers and flavour enhancers. Compared to Type I sourdough, microbiota in Type II sourdough can be better controlled by addition of starter culture, which leads to improved and reproducible bread quality. *Lactobacillus* strains isolated from Type II sourdough such as *L. reuteri* are usually subjected to fermentation for extended periods at low pH, and exhibit high acid resistance (Gänzle, 2005; Meroth et al, 2003). The range of pH in Type I and II sourdoughs can be from 3.5-4.3 in wheat sourdough (Collar et al, 1994; Wehrle and Arendt, 1998; Thiele et al, 2002; Stromeck et al, 2011).

During sourdough fermentation, amino acids are continuously released from protein by cereal enzymes, so the ability to use amino acids for acid resistance is of particular importance for Type II sourdough strains. Amino acids based acid resistance systems including arginine and glutamate dependent systems have been well understood in both gram-positive bacteria and gram-negative bacteria (Cotter

and Hill 2003; Foster 2004). There are several pathways for acid resistance in *Lactobacillus* strains, including arginine deiminase (ADI) pathway and glutamate decarboxylase (GAD) activity (Rollan et al, 2003; Park and Oh, 2007). ADI allows utilization of arginine under anaerobic conditions in *Bacillus licheniformis* (Maghnouj et al, 1998). Enzymes of ADI pathway were present in all *Leuconostoc* strains tested (Liu et al, 1995). Arginine is released by cereal proteinase during sourdough fermentation and converted by ADI to citrulline, and further converted to ornithine by ornithine transcarbamoylase (OTC) and carbamate kinase (CK) (Rollan et al, 2003). Metabolism of one mole of arginine to ornithine produces 1 mole of ATP and 2 moles of ammonia, with consumption of 2 moles of protons (Champomier Verges et al, 1999; Budin-Verneuil et al, 2004). Expression of ADI in *Lactobacillus* strains can be induced by energy depletion or addition of arginine (Rollan et al, 2003; Champomier Verges et al, 1999; Cunin et al, 1986). Regulation of ADI has been studied in *Lactobacillus leichmannii* and *Lactobacillus sakei* (Manca de Nadra et al, 1986), *Lactobacillus plantarum* (Arena et al, 1999; Spano et al, 2004), and *Lactobacillus buchneri* (Manca de Nadra et al, 1981). The ADI system is quite effective even under acidic conditions, as addition of arginine promoted growth of *Lactobacillus reuteri* during survival test at pH 3.0 (Rollan et al, 2003).

GAD is widely distributed among eukaryotes and prokaryotes, and has also been reported as an effective system against acid stress (Nomura et al, 1999; Ueno 2000; Park and Oh, 2007; Sanders et al, 1998a). GAD catalyzes the conversion of glutamate to γ-aminobutyric acid (GABA), releasing CO_2 and ATP (Higuchi et al,

1997). Sonicated and heated spores of *Bacillus megaterium* showed high GAD activities (Foerster and Foerster, 1973). Rye malt sourdough with the addition of wheat gluten fermented by *L. reuteri* resulted in accumulation up to 90 mmol/kg dry matter (Stromeck et al, 2011).The optimum pH for GAD in *E. coli* was pH 4.4 (Fonda 1985, as cited in Hiraga et al, 2008). Similar optimum pH values of GAD have been observed in *Streptococcus salivarius* ssp. *thermophilus* and *L. brevis* (Yang et al, 2008; Hiraga et al, 2008). Su et al (2011) showed that GAD effectively protected *L. reuteri* from acidic conditions (pH 2.5) during incubation in phosphate buffer for 24 hours.

In addition to ADI and GAD, glutaminase has the potential in promoting survival under acidic conditions. Glutaminase belongs to the amidohydrolase enzyme group, which specifically deaminate glutamine into glutamate by hydrolytic activity (Nandakumar et al, 2003). Glutaminase was biochemically characterized in *L. reuteri*, and addition of glutamine resulted in higher optical density when culture was grown in mMRS media containing nigericine⁄ valinoymcin (Vermeulen et al, 2007). This conversion is of particular interest in wheat, rye and barley fermentation, as prolamin in those cereals can contain more than 30% of glutamate/glutamine (Lásztity 1983). Glutaminase is well distributed among prokaryotes and eukaryotes and glutaminase activity are strain specific (Siragusa et al, 2007; Weingand-Ziade et al, 2003; Vermeulen et al, 2007). Biochemical characterization of glutaminase activity has been studied in *Lactobacillus rhamnosus* (Weingand-Ziadé et al, 2003). *L. sanfranciscensis* and *L. reuteri* have also been reported to possess glutaminase activity (Vermeulen et al, 2007).

Various optimum pH values of glutaminases have been reported. *E. coli* has a glutaminase with an optimum pH of 5.0, whereas glutaminase in *L. rhamnosus* has an optimum pH of 7.0 (Weingand-Ziadé et al, 2003). In *L. reuteri*, the optimum pH of glutaminase was between 5.0 and 6.0 (Vermeulen et al, 2007).

Arginine and glutamate dependent acid resistance systems are important strategies for microorganisms to survive when passing through human stomach (Richard and Foster, 2004). Glutamine also has the potential in promoting survival of microorganisms under acid stress, but utilization of glutamine in response to acid stress has not yet been reported. *L. reuteri* 100-23 was isolated from a rodent forestomach, which contained putative genes encoding glutaminase, ADI and GAD in its genome. Strains of *L. reuteri* do not only inhabitat sourdough but also gastrointestinal tract of pigs, chicken and rodents (Walter 2008), which suffers acid stress when passing through stomach. Both ADI and GAD have been well-characterized biochemically and genetically in strains of *Lactobacillus* (Arena et al, 2002; Brown et al, 2008; Park and Oh, 2007; Hiraga et al, 2008; De Angelis 2002). Glutaminase has been characterized biochemically in lactic acid bacteria (Weingand-Ziadé et al, 2003), but genetic characterization remains unclear. Additionally, it is of interest to understand whether the three acid resistance systems in *Lactobacillus* spp. are redundant or complementary under different stress conditions. Inducible gene expression in response to acid stress is an important survival strategy widely used by microorganisms (Rollan et al, 2003; Brown et al, 2008; Hersh et al, 1996). In this study, we indentified three putative glutaminases in *L. reuteri* 100-23, and examined the role of glutaminase in

reponse to acid stress. A combination of knock-down studies and quantification of gene expression were used in this study to identify the relevant mechanism of acid resistance under different conditions. Additionally, this study also offered comparative view of gene expression of *adi*, *gadB*, *gls1*, *gls2*, and *gls3* under acid resistance under same conditions.

4.2. Materials and methods.

4.2.1. Media, strains and growth conditions.

Lactobacillus reuteri 100-23, *L.reuteri* 100-23 Δ*gls1*, *L.reuteri* 100-23 Δ*gls2*, *L.reuteri* 100-23 Δ*gls3*, and *L.reuteri* 100-23 Δ*gadB* strains were grown in mMRS as previously described (Zhang and Gänzle, 2010; Su et al, 2011). Ten or 20 mM of arginine, glutamine, or glutamate were added and the pH of the media was adjusted to 6.0 as indicated. One mL of culture was grown overnight, then 50 µL of culture was used to inoculate 5mL medium containing 23 mM maltose and 11.5 mM fructose and samples from exponential phase (5 hours, OD_{600}=0.5) and late stationary phase (48 hours, OD_{600}=1.6) were taken. All incubations were conducted at 37 ºC under anaerobic conditions.

4.2.2. Generation and verification of *gls* and *gadB* mutants of *L. reuteri* 100-23.

L. reuteri 100-23 Δ*gls1*, *L. reuteri* 100-23 Δ*gls2*, *L. reuteri* 100-23 Δ*gls3* and *L. reuteri* 100-23 Δ*gadB* were constructed and verified by Marcia Shuwei Su and Sabine Schlicht using a deletion strategy described by Su et al, 2011. Primers used in generation and confirmation of all mutants are listed in Table 4.1.

4.2.3. Culture conditions for RNA isolation of *L. reuteri* 100-23 at exponential phase and stationary phase in mMRS media.

To compare the differences in expression of *gls1*, *gls2*, *gls3*, *adi*, and *gadB* in *L.*

Table 4. 1. List of primers used in RT-qPCR and mutant generation.

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
Δgls1	5' fragment Forward	AAC TGC AGA AAA GCT TGG ACA ACC C
	5' fragment Reverse	GGA ATT CTT ATT TAA GAT CCA AAG TAAT CAC CTC
	3' fragment Forward	GGA ATT CTT TCA GTA CTA ATA ATT AAG GTC CAA
	3' fragment Reverse	CGG GAT CCG CAT GTG CTG AAA ATT G
	Confirmation Forward	CCT TTA TCA ACC ATC AGC T
	Confirmation Reverse	AGC TGG TGT GCT ACT TT
Δgls2	5' fragment Forward	AAC TGC AGG GGA TTG TAA CTT GAA ATT AAC
	5' fragment Reverse	GAA GAT CTT CAT TCT TGA ATT GCG TCA TTA AG
	3' fragment Forward	GAA GAT CTA GGT ACT AGT TGC AAA TAT TCG C
	3' fragment Reverse	CGG GAT CCG ATA TTC AGC AGT CGA AAG
	Confirmation Forward	GCC AAA TAT CTG CTG ATC G
	Confirmation Reverse	AAC AGC GTT TGT TCC AA
Δgls3	5' fragment Forward	CGG GAT CCT TGC CGA TGC ATT AAC
	5' fragment Reverse	GCT CTA GAC TAT TGC TCT AAT TTT TGC ATC GT
	3' fragment Forward	GCT CTA GAT TAG AAT TAG TAG TTT AAT AAA AGC G
	3' fragment Reverse	AAC TGC AGG GAA ACG CAG ATG AGA G
	Confirmation Forward	AGA GCG GGG TAT TTC G
	Confirmation Reverse	GCT GGT TGG GTA AAA GTT

reuteri 100-23 between exponential phase and stationary phase in presence or absence of 10 mM glutamine, overnight cultures of *L. reuteri* 100-23 were cultured as previously described. Fifty microlitres of overnight culture were inoculated into mMRS media, pH 6.0, containing 23 mM of maltose and 11.5 mM of fructose. Cultures were taken at exponential phase and stationary phase and gene expression of *gls1*, *gls2*, *gls3*, *adi*, and *gadB* were determined by Reverse Transcription quantitative PCR (RT-qPCR) as described below.

4.2.4. Culture conditions for RNA isolation of *L. reuteri* 100-23 at exponential phase after short acid stress in mMRS media.

To further determine whether the inducible expressions of *gls1*, *gls2*, *gls3*, *adi*, and *gadB* of *L. reuteri* 100-23 were triggered by acid stress, one milliliter of culture was grown to exponential phase as described above and pellets were collected by centrifugation and subjected to acid stress (pH 3.5 for 40 minutes) in mMRS media. Cultures incubated at pH 6.0 in mMRS media were used as control. Expression level of *gls1*, *gls2*, *gls3*, *adi*, and *gadB* were determined by RT-qPCR as described below.

4.2.5. Culture conditions for RNA isolation of *L. reuteri* 100-23 Δ*gadB* and *L. reuteri* 100-23 Δ*gls3* at exponential phase in mMRS media.

To understand the differences of gene expression in *L. reuteri* 100-23 Δ*gadB* and Δ*gls3* strains compared to wild type strains, cultures of *L. reuteri* 100-23 Δ*gadB* and Δ*gls3* strains were grown to exponential phase and stationary phase in

absence and presence of 10 mM glutamine, respectively. Expression of *gls3* and *adi* in Δ*gadB* culture and gene expression of *gls1*, *gls2*, *gls3*, *adi*, and *gadB* in Δ*gls3* culture were quantified by RT-qPCR as described below.

4.2.6. Survival of *L. reuteri* 100-23 and *L. reuteri* 100-23 Δ*gadB* after acid stress at pH 3.5 for 24 h or pH 2.5 for 10 h in buffer.

To determine which of the three enzymes were responsible for acid stress at two different pH values, the survival of *L. reuteri* 100-23 after acid stress in 100 mM lactate buffer (pH 3.5) for 24 hours and 100 mM phosphate buffer (pH 2.5) for 10 hours were compared. Use of lactate buffer at pH 3.5 and phosphate buffer at pH 2.5 aimed to imitate sourdough and the mammalian stomach, respectively. Overnight cultures were grown as described above and cell pellets were collected by centrifugation and resuspended in buffers containing 20 mM arginine, glutamine or glutamate, and incubated at 37 ºC for 24 hours at pH 3.5 or 10 hours at pH 2.5. Serial dilutions were plated on mMRS agar media for cells counts. Results are expressed in mean value ±error from two independent experiments.

The survival of *L. reuteri* 100-23 Δ*gadB* and *L. reuteri* 100-23 Δ*gls3* was analyzed at pH 2.5 in 100 mM phosphate buffer to understand the acid resistance for mutant strains. Overnight culture was grown as described above and cell pellets was collected by centrifugation and resuspended in buffers containing 20 mM arginine, glutamine or glutamate, and incubated at 37 ºC for 10 h at pH 2.5. Serial dilutions were plated on mMRS agar media for cells counts. Two

independent experiments were conducted and results showed one of the representative experiments.

4.2.7. Culture conditions for RNA isolation of *L. reuteri* 100-23 in wheat sourdough.

To understand the expression of *gls1*, *gls2*, *gls3*, *adi*, and *gadB* in wheat sourdough system, cells pellets from one milliliter of overnight culture were collected by centrifugation and resuspended with 10 mL tap water and then mixed with 10 g whole wheat flour (obtained from local market). Dough was incubated at 37 ºC and 0.5 g sourdoughs of exponential phase (5 h) or stationary phase (48 h) were added into 3 mL of RNA protect (Qiagen, U.S.A). Sample was mixed and incubated at room temperature for 10 min, and then subjected to centrifugation at 400 x *g* for 10 min. The supernatant was centrifuged at 600 x g for 10 min and supernatant was collected. Finally, supernatant was centrifuged at 5000 x g and the pellet was collected and stored at -80 ºC before RNA isolation. Protocol for RNA isolation was described below. Expression levels of *gls1*, *gls2*, *gls3*, *adi*, and *gadB* were determined as indicated below.

4.2.8. Analysis of metabolites with High Performance Liquid Chromatography (HPLC).

Concentrations of amino acids including arginine, citrulline, ornithine, glutamine, and glutamate were determined with HPLC as previously described (Sedgwick et al, 1991).

4.2.9. RNA isolation and quantification of mRNA of *gls1*, *gls2*, *gls3*, *adi*, and *gadB* expression in *L. reuteri*, and *L. reuteri* 100-23 Δ*gadB* and *L. reuteri* 100-23 Δ*gls3* strains.

Cultures were grown to exponential or stationary phase as indicated above and two or six volumes of RNA protect were added to 1 mL of culture or 0.5 g of sourdough, respectively, to maintain the integrity of RNA. Cultures were centrifuged to remove the supernatants and cells pellets were stored at -80 ºC before isolating RNA. RNA was isolated using Trizol reagent according to manufacture's instruction (Molecular Research Center, Inc, Cincinnati, U.S.A) then subjected to DNase treatment and reverse transcription as previously described (Schwab and Gänzle, 2006). Genes of *gls1*, *gls2*, *gls3*, *adi*, and *gadB* were amplified by specific primers (Table 4.1) and quantified with PCR on a 7500 Fast Real Time-PCR System (Applied Biosystems, USA). Product size was confirmed by agarose gel electrophoresis. Primers targeting all genes were designed according to sequence of *L. reuteri* 100-23 genome (Integrated microbial genome). Appropriate transcripts were ensured by analyzing melting curve and using DNase treated RNA as control during RT-qPCR. Phosphoketolase was used as a reference gene and Cycle Threshold (CT) of the target and reference genes were used to calculate the normalized gene expression:

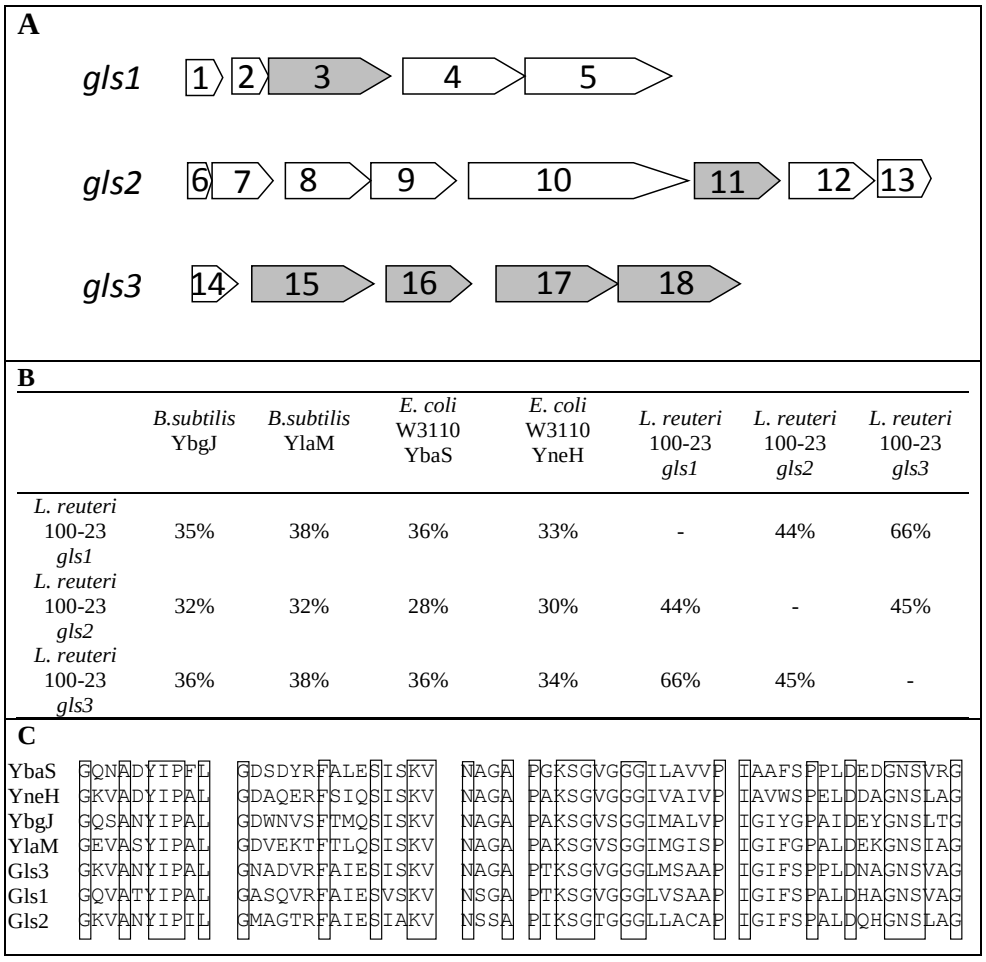
$$N = \frac{E_{target}^{\Delta CT_{target}(Control-sample)}}{E_{reference}^{\Delta CT_{reference}(Control-sample)}}$$ (Pfaffl 2001). E is efficiencies of PCR reactions and PCR efficiencies of *pho*, *gls1*, *gls2*, *gls3*, *adi*, and *gadB* were

determined as 1.90, 1.89, 1.90, 1.91, 2.0, and 2.0, respectively. Gene expression of *pho* in *L. reuteri* 100-23 strain grown in absence of glutamine was used as the control (Schwab and Gänzle, 2006). Each sample was analyzed in duplicate and results from mean value of two independent experiments are shown.

4.3. Results.

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

To indentify genetic determinants of glutamine deamidation, putative glutaminases were identified in the genome of of *L. reuteri* 100-23 (Figure 4.1). Genomic neighborhood of *gls1*, *gls2*, and *gls3* showed that *gls3* was adjacent to *gadB* and two glutamate/GABA antiporters. Both *gls1* and *gls2* are not adjacent to any *gadB* or glutamate/GABA antiporter (Figure 4.1A). Alignment of protein sequences for the three glutaminases in *L. reuteri* 100-23 showed 44% - 66% homology, while alignment of protein sequences of three glutaminases in *L. reuteri* 100-23 with four characterized glutaminases in *E. coli* W3110 and *B. subtilis* by Brown et al (2008) showed 28% - 38% of homology (Figure 4.1B). Alignment of conversed catalytic residues of glutaminases showed that glutaminases in *L. reuteri* 100-23 contained most of conserved catalytic residues stated in glutaminases in *E. coli* W3110 and *B. subtilis* (Figure 4.1C). In all, both comparison of homology and catalytic residues of three glutaminases in *L. reuteri* 100-23 and four glutaminases in *E. coli* W3110 and *B. subtilis* by indicated that *gls1*, *gls2*, and *gls3* of *L. reuteri* 100-23 were glutaminases.



B

	B.subtilis YbgJ	*B.subtilis* YlaM	*E. coli* W3110 YbaS	*E. coli* W3110 YneH	*L. reuteri* 100-23 *gls1*	*L. reuteri* 100-23 *gls2*	*L. reuteri* 100-23 *gls3*
L. reuteri 100-23 *gls1*	35%	38%	36%	33%	-	44%	66%
L. reuteri 100-23 *gls2*	32%	32%	28%	30%	44%	-	45%
L. reuteri 100-23 *gls3*	36%	38%	36%	34%	66%	45%	-

C

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

Figure 4.1. Homology of protein sequence alignment between three glutaminases in *L. reuteri* 100-23 and four glutaminases characterized by Brown et al, 2008. Accession number of *gls1*, *gls2*, and *gls3* are 639134920, 639133703, and 639134641, respectively. Panel A. Genic loci coding for glutaminases in *L. reuteri* 100-23. Genes coding for glutamine transport or metabolism are shaded in grey. Open reading frames were annotated as follows (Integrated microbial genomes: http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=TaxonDetailandpage=taxonDetailandtaxon_oid=638341112): 1: Thioredoxin-related, 2: Conserved hypothetical protein, **3: Glutaminase**, 4: Aldo/keto reductase, 5: Glycosyl transferase, 6: Unknown, 7: Heat shock protein, Metallo peptidase, 8: N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase, 9: Helicase-like, 10: Conserved hypothetical protein, **11: Glutaminase**, 12:DltD, central region(D-alanine transfer protein), 13: D-alanyl carrier protein, 14: Ribosomal protein S4:RNA-binding S4, **15: glutamate:gamma-aminobutyrate antiporter, 16: Glutaminase, 17: Glutamate: GABA antiporter, 18: Glutamate decarboxylase**. Panel B. Homology the three glutaminases in *L. reuteri* 100-23 to glutaminases in *E. coli* and *B. subtilis* (Brown et al, 2008). Panel C. Conserved residues in glutaminases of *L. reuteri* 100-23, *B. subtilis* and *E. coli*.

4.3.2. Gene expression of *L. reuteri* 100-23 at exponential phase and stationary phase in mMRS media, after short acid stress and in sourdough.

Gene expression of *gls1*, *gls2*, *gls3*, *adi*, and *gadB* in *L. reuteri* 100-23 in stationary phase compared to exponential phase were determined (Table 4.2). Compared to exponential phase, *gls3* was over-expressed in the stationary phase, whereas the corresponding differences in expression of *gls1* and *gls2* was similar. Similar level of over-expression of *gadB* was shown compared to *gls3*. Expression of *adi* was repressed in the stationary phase compared to exponential phase. Addition of glutamine resulted in over expression of only *gls3* in stationary phase.

To determine whether over expression of genes at stationary phase compared to exponential phase was due to acid stress, gene expression levels of *gls1*, *gls2*, *gls3*, *adi*, and *gadB* in *L. reuteri* 100-23 were analyzed after short acid stress in mMRS media as shown in Table 4.2. Similar levels of gene over-expression of *gls3* and *gadB* were observed as a confirmation of gene over-expression in stationary culture. Compared to the controls, expression levels of *gls1* and *gls2* were similar after exposure to short acid stress. Gene expression indicated that *gls3* is the most highly expressed gene among three putative glutaminases for acid resistance.

Substrate availability in wheat sourdough is different from that in mMRS medium. To verify expression of *gls1, gls2, gls3, adi,* and *gadB* in wheat sourdough system, gene expression in sourdough was compared with gene expression of exponential phase cells grown in mMRS medium (Table 4.2). In addition to noticeable over-expression of *gls2*, a high level of over-expressions of *gls3* and *gadB* was

observed in stationary phase in sourdough. The level of over-expression of *gls3* and *gadB* were comparable. Metabolites produced from sourdough fermentation were analyzed (Figure 4.3). Main metabolites produced from ADI pathway and GAD activity, including arginine, ornithine, glutamine, and glutamate, were still present after fermentation, indicating that the substrate was not the limiting factor in sourdough.

Table 4. 2. Gene expression of *L. reuteri* 100-23 at exponential phase and stationary phase in mMRS medium, after short acid stress, and in sourdough. Results shown are comparison with gene expression of exponential phase cells of *L. reuteri* 100-23 grown in the absence of glutamine in mMRS media. Results from mean value of two independent experiments are shown.

	mMRS	mMRS	mMRS+Gln		Sourdough	
	48h	5h+40min at pH 3.5	5h	48h	5h	48h
gls1	0.4±0.1	1.8±0.2	0.8±0.2	0.6±0.3	0.3±0.1	0.5±0.2
*gls*2	0.9±0.2	1.4±0.3	1.7±0.1	1.6±0.6	0.2±0.1	3.5±1.5
*gls*3	4.3±1.3	6.1±0.8	2.0±1.0	8.9±0.6	3.7±0.9	233.6±110.4
adi	0.1±0.0	0.5±0.1	n.d.	n.d.	0.1±0.0	0.8±0.3
gadB	7.6±1.6	7.0±1.0	n.d.	n.d.	0.9±0.2	369.9±75.8

4.3.3. Gene expression of *L. reuteri* 100-23 Δ*gadB* and *L. reuteri* 100-23 Δ*gls3* strains at exponential phase in mMRS media.

To determine whether disruption of one acid resistance gene will be compensated by over-expression of other acid resistance genes, expression levels of *gls1*, *gls2*,

gls3, *adi*, and *gadB* in *L. reuteri* 100-23, *L. reuteri* 100-23 Δ*gadB* and *L. reuteri* 100-23 Δ*gls3* strains were determined (Table 4.3). Deletion of *gadB* resulted in over-expression of *adi* and *gls3*, whereas deletion of *gls3* resulted in over-expression of *gls1*, *gls2* and *gadB* compared to the gene expression in the wild type strains.

Table 4. 3. Gene expression of *L. reuteri* 100-23 Δ*gadB* and *L. reuteri* 100-23 Δ*gls3* at exponential phase and stationary phase. Results were shown in comparison to gene expression of *L. reuteri* 100-23 grown in absence of glutamine at exponential phase. Results from mean value of two independent experiments are shown

		5h	48h
Δ *gadB*	*adi*	0.6±0.4	4.5±1.2
	gls3	7.4±0.9	28.4±10.4
Δ *gls3*	*gls1*	3.8±1.2	3.7±0.9
	gls2	7.1±1.8	6.9±0.9
	adi	0.2±0.0	0.2±0.1
	gadB	0.5±0.1	4.7±1.0

4.3.4. Survival of *L. reuteri* 100-23 and *L. reuteri* 100-23 Δ*gadB* after acid stress at pH 3.5 for 24 h or pH 2.5 for 10 h in buffers.

Ability of culture of *L. reuteri* 100-23 to survive under acid stress was conducted in lactate buffer at pH 3.5 and phosphate buffer at pH 2.5 to imitate the pH of sourdough fermentation during stationary phase and pH in rodent stomach

respectively (Arnold and Kaspar, 1995). Survival of *L. reuteri* 100-23 at pH 3.5 for 24 h and pH 2.5 for 10 h in buffers were compared to determine which amino acid is responsible for acid resistance at different pH values (Figure 4.2). At pH 3.5, culture incubated in presence of 20 mM arginine was 100-fold more acid resistant compared to the culture incubated in buffer only, indicating that ADI pathway is effective in acid resistance at pH 3.5. The culture incubated in presence of 20 mM glutamine and glutamate was less acid resistant than the culture incubated in presence of arginine. At pH 2.5, the culture incubated in presence of 20 mM glutamine or glutamate was more acid resistant than the culture incubated in buffer only. Futhurmore, the culture incubated in presence of 20 mM glutamine were more acid resistant than the culture incubated in presence of 20 mM arginine. In conclusion, data demonstrated that at pH 3.5, the ADI pathway was more effective than glutaminase and GAD pathway for acid resistance, whereas at pH 2.5, glutaminase pathway is the most effective pathway for acid resistance.

Survival of *L. reuteri* 100-23 Δ*gadB* under acid stress was conducted in phosphate buffer at pH 2.5 (Figure 4.2). In absence or presence of glutamine, no difference in bactericidal effect after acid stress was observed among three glutaminase mutants. However, higher cells counts was shown in all glutaminase mutants incubated in presence of glutamine compared to corresponding mutants incubated in absence of glutamine, confirming that glutaminase promoted culture survival at pH 2.5 (data not shown). *L. reuteri* 100-23 Δ*gadB* incubated in presence of glutamine showed higher cells counts compared to culture of *L. reuteri* 100-23

Δ*gadB* incubated in absence of glutamine and *L. reuteri* 100-23 Δ*gadB* incubated in presence of glutamate, demonstrating that glutaminase activity in additional to GAD activity is responsible for acid resistance at pH 2.5.

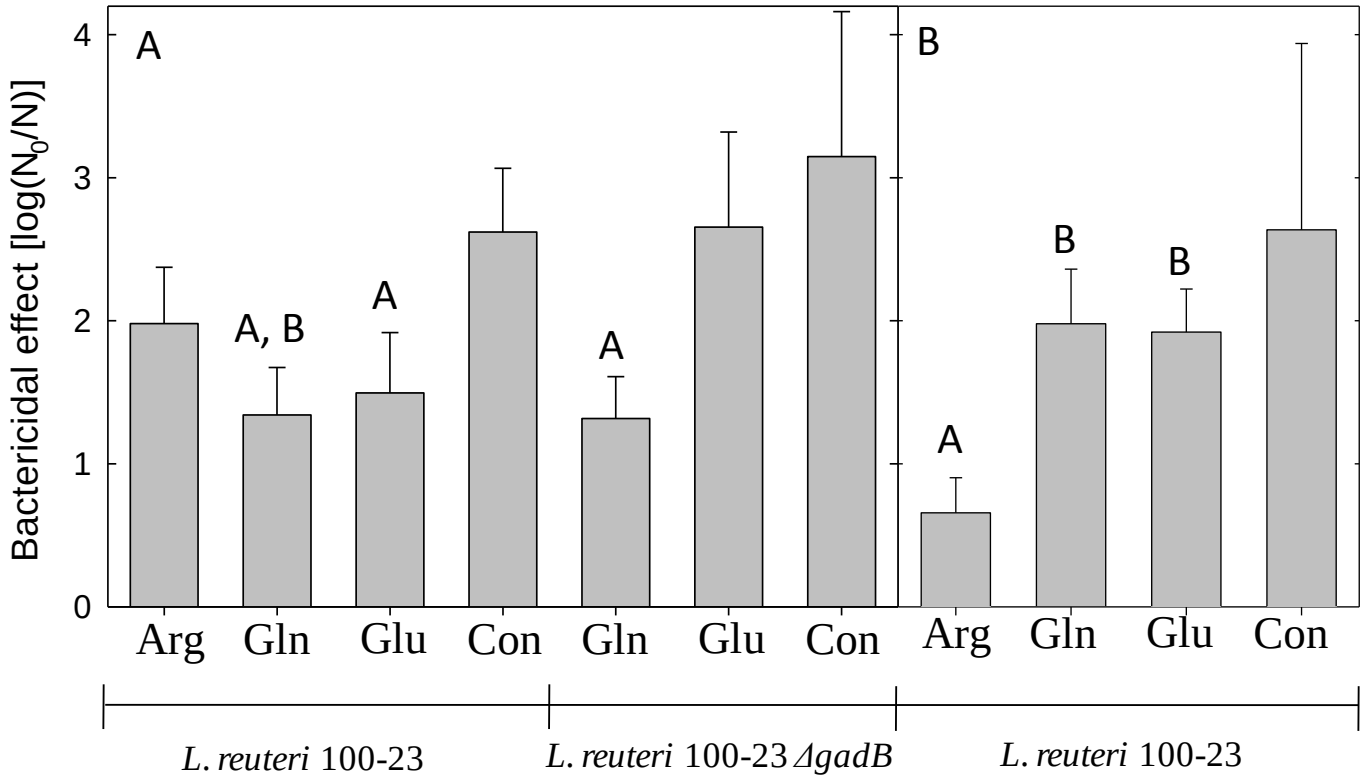

Figure 4.2.Survival of *L. reuteri* 100-23 or *L. reuteri* 100-23 Δ*gadB* after acid stress in buffer containing 20 mM arginine, glutamine, or glutamate at pH 2.5 for 10 h (Panel A) or at pH 3.5 for 24 h (Panel B). Controls are survival of *L. reuteri* 100-23 or *L. reuteri* 100-23 Δ*gadB* after acid stress in buffer only. Results are the mean of four independent experiments, and significance of the results are analyzed by t-Test ($P<0.05$). A: comparison to survival of *L. reuteri* 100-23; B comparison to survival of *L. reuteri* 100-23 in presence of arginine. N_0: initial cells count (cfu/ml); N: cells count after treatment (cfu/ml).

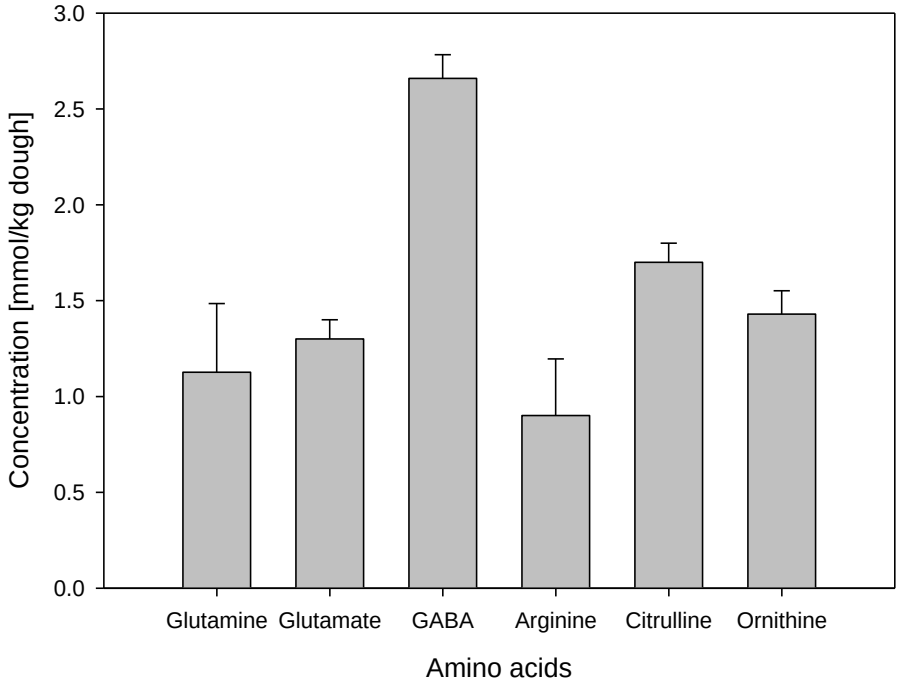

Figure 4.3. Concentration of amino acids in wheat sourdough after fermentation by *L. reuteri* 100-23 for 48 h. Results are the mean of two independent experiments.

4.4. Discussion.

Amino acid-based acid resistance systems, especially arginine and glutamate, are important surviving strategies of microorganisms and widely distributed in bacteria (Cotter and Hill 2003; Foster 2004). The mechanism can be release of ammonia, CO_2 or production of ATP. Positive effect of glutamate production and increased pH was reported in *L. rhamnosus* during fermentation, indicating glutaminase activity (Weingand-Ziadé et al, 2003). Addition of 75 mM glutamine resulted in improved growth in *L. reuteri* and *L. sanfranciscensis* at pH 5.0, suggesting conversion of glutamine to glutamate improved acid tolerance (Vermeulen et al, 2007). This study demonstrated that glutaminase greatly promoted culture survival and indicated that glutaminase was more effective at pH 2.5 compared to pH 3.5.

Arginine enhances acid survival of both gram-negative and gram-positive bacteria (Ryan et al, 2009; Ryan et al, 2008; Richard and Foster, 2004; Curran et al, 1995). The ADI pathway is an important system for acid resistance in *Listeria monocytogenes* and contributes both to growth and survival in acid conditions (Ryan et al, 2009; Ryan et al, 2008). During an acid challenge at pH 2.5, addition of arginine increased the pH of medium where *E. coli* was grown (Richard and Foster, 2004). The concentration of arginine decreased with time and excretion of citrulline was reported in *Oenococcus oeni* (Mira de Orduna et al, 2001). D value at pH 3.5 of acid adapted streptococci culture was 3 times higher when culture was incubated in presence of 10 mM ADI compared to culture incubated in absence of arginine (Curran et al, 1995). Many *Lactobacillus* strains can convert

arginine into ornithine via ADI pathway (Thiele et al, 2002). Addition of arginine promoted better survival during storage of sourdough at 7 °C for 12 hours, during incubation of *L. sakei* in chemically defined medium for 48 hours, during acid challenge at pH 3.0 for 1 hour in *L. reuteri* (De Angelis et al, 2002; Champomier Vergès et al, 1999; Budin-Verneuil et al, 2004; Rollan et al, 2003). This study confirmed the role of ADI pathway in promoting acid resistance.

GAD protection from acid stress under low pH has been widely used in microorganisms such as in lactic acid bacteria, *Listeria* and *E. coli* (Cotter and Hill 2003; Foster 2004). GAD greatly promoted survival of *Listeria monocytogenes*in gastric fluid (Cotter et al, 2001). Acid challenge at pH 2.0 for 2 hours in media for *E. coli* showed that the addition of glutamate resulted in higher percentage of survival compared to addition of arginine (Lin et al, 1996). Similar results had been obtained during an acid challenge at pH 2.0 for 1 hour for *E. coli*, which resulted in much higher survival in presence of glutamate than a culture exposed to arginine (Hersh et al, 1996). Although acid challenge in *E. coli* at pH 2.5 was usually conducted for 1 to 2 hours, as *E. coli* survived when passing through the acidic stomach (Arnold and Kaspar, 1995), survival under extreme pH for long period has been reported in *E. coli*, where percentage of survival after 10 hours at pH 2.0 can be as high as 10% (Diez-Gonzalez and Russell, 1999). The period of acid challenge in this study was extended to 10 hours in order to obtain obvious differences between different treatments. One possible explanation of ability of *L. reuteri* 100-23 to survive for such long period is that *L. reuteri* 100-

23 was isolated from the rodent forestomach, where they tolerated acidic environment as low as pH 2.9 (Ward and Coates, 1987).

Stromeck et al (2011) reported that glutamate accumulated during sourdough fermentation by several *Lactobacillus* strains. Kinetic measurement of amino acid level during fermentation by *L. reuteri* 100-23 wild type strain and Δ*gadB* mutant showed that glutamate released into sourdough was much higher in wild type strain than in *L. reuteri* 100-23 Δ*gadB*, indicating that glutamine but not glutamate is the real substrate for the conversion during acid stress (Su et al, 2011). Importance of glutaminase activity in acid resistance has been speculated in several studies but not confirmed. This study offers the first genetic characterization of glutaminase in LAB. Genomic neighborhood showed that only *gls3* was located in the same operon with *gadB* and GABA antiporter, indicating the potential role of *gls3* in conversion of glutamine to GABA through glutamate. Analysis of gene expression of *L. reuteri* 100-23 in stationary phase, gene expression of *L. reuteri* 100-23 after short acid stress, and gene expression of *L. reuteri* 100-23 in sourdough all showed comparable level of over-expression of *gls3* and *gadB*. Survival of *L. reuteri* 100-23 Δ*gadB* at pH 2.5 demonstrated that addition of glutamine greatly promoted acid resistance compared to culture incubated without glutamine. Gene expression in *L. reuteri* 100-23 Δ*gadB* also confirmed that deletion of *gadB* is compensated by over-expression of *gls*3 and *adi*, indicating the important role of glutaminase activity in acid resistance.

L. reuteri is highly competitive in Type II sourdough (Meroth et al, 2003). Multiple amino acid-based acid resistance systems are of high importance for *L.*

reuteri, as cultures used for Type II wheat sourdough fermentation can be subjected to acid stress as low as pH 3.5 for several days (Böcker et al, 1995; Vogel et al, 1999). Utilization of arginine, glutamine and glutamate all contribute to acid resistance. Glutamate dependent acid resistance system improved competitiveness of *L. reuteri* 100-23 in sourdough (Su et al, 2011). Acid challenge in this study showed promotion of survival by arginine or glutamine addition at both pH 3.5 and 2.5. Redundant acid resistance systems in *L. reuteri* promoted acid stress under different pH values leading to higher yield of flavour enhancer used in sourdough bread.

Lactobacilli strains dominate microbiota of gastrointestinal tract (GI) of rodent, pig and chicken. *L. reuteri* inhabits the forestomach of rodent and esophagus of pig, where it suffers acid stress as low as pH 2 when passing through the GI tract (Walter 2008). EPS produced by *L. reuteri* play an important role during colonization. Levan produced by in *L. reuteri* inhibited hemagglutination of entertoxigenic *E. coli* (Wang et al, 2010). Production of the exopolysaccharides reuteran or levan promoted acid survival of *L. reuteri* TMW1.106 and 100-23, respectively (Kaditzky et al, 2008; Sims et al, 2011). Redundancy of acid resistance systems in *L. reuteri* 100-23 can be likely due to adaptation of the acidic environment when passing through the GI tract (Walter 2008).

Acid tolerance of arginine decarboxylase and GAD has been compared in *E. coli* (Richard and Foster, 2004), but no comparative study has been conducted previously under same conditions for the acid resistance in lactobacilli. Current

study filled the blank and offered a novel view of how *L. reuteri* 100-23 reacts to acid stress at different pH values.

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Chapter 5: General discussion and conclusion

5. 1. Discussion and conclusion.

This thesis developed a better understanding of the ecological role of metabolic pathways of strains of lactobacilli used in wheat sourdough**.** Specifically, three individual pathways of strains of lactobacilli were studied. First, propionic acid was produced during cofermentation by *L. buchneri* and *L. diolivorans* to prevent spoilage in bread. Propionate formation was observed in cofermentation with *L. buchneri* and *L. diolivorans* in modified MRS broth as well as sourdough with low, medium and high ash contents. Sourdough fermented with *L. buchneri* and *L. diolivorans* (experimental sourdough) was used in bread making and its effect on fungal spoilage was compared to traditional sourdough or propionate addition. Bread slices were inoculated with *Aspergillus clavatus*, *Cladosporium* spp., *Mortierella* spp. or *Penicillium roquefortii*. The use of 20% experimental sourdough inhibited growth of three of the four molds for more than 12 days. The use of 10% experimental sourdough deferred growth of two of four molds by 1 day. Secondly, metabolism of α-KG was identified in *L. sanfranciscensis* and *L. reuteri* in modified MRS and sourdough. *L. sanfranciscensis* and *L. reuteri* were grown with additional α-KG in mMRS and in wheat sourdough. In mMRS, α-KG was used as an electron acceptor and quantitatively converted to 2-OHG by both organisms. Production of 2-OHG was identified by HPLC and confirmed by GC. Crude cells extracts of *L. sanfranciscensis* and *L. reuteri* grown with or without α-KG exhibited OHG-dehydrogenase acitivity of 6.3±0.3, 2.3±0.9, 1.2±0.2, and 1.1±0.1 mM NADH (min x mg protein)$^{-1}$, respectively. The presence of phenylalanine and citrate in addition to α-KG partially redirected the use of α-KG

from electron acceptor to amino group acceptor. In wheat sourdoughs, α-KG was predominantly used as electron acceptor and converted to 2-OHG by both organisms. The use of α-KG as electron acceptor in hetero-fermentative lactobacilli impacts the formation of flavour volatiles through the transamination pathway. Finally, genes encoding the enzymes responsible for metabolism of glutamine to glutamate by *Lactobacillus reuteri* 100-23 were characterized at the genetic level. In addition, the effect of glutaminase activity on acid resistance was compared with ADI and GAD in buffer. Inducible gene expression for improved acid resistance is an important survival strategy for *Lactobacillus*. *L. reuteri* 100-23 contains three glutaminases (Gls1, Gls2, and Gls3), one ADI, and one GAD. Survival of *L. reuteri* 100-23 at pH 3.5 for 24 hours and pH 2.5 for 10 hours in buffers were compared to determine which enzymatic pathway was responsible for acid resistance at different pH values. Results showed that under pH 3.5, ADI pathway is more involved in acid resistance. Levels of over-expression in those five genes during stationary phase compared to exponential phase were studied in media for *L. reuteri* 100-23 wild type, Δ*gadB* and Δ*gls3* strains as well as in wheat sourdough for *L. reuteri* 100-23. Results in media for *L. reuteri* 100-23 wild type strain showed that *gls3* was over-expressed for acid resistance among the three glutaminase genes; compared to exponential phase, levels of gene over-expression in stationary phase was enhanced by addition of glutamine. Analysis of gene expression in Δ*gadB* and Δ*gls3* strains showed that deletion of *gadB* resulted in over- expression of *adi* and *gls3*, whereas deletion of *gls3* showed over-expression of *gls1*, *gls2* and *gadB* compared to wild type

strains. Gene expression in wheat sourdough showed over-expression of all above five genes.

Previous chapters discussed the role of propionate produced by cofermentation of *L. buchneri* and *L. diolivorans* as antifungal preservative to extend bread shelf life. Propionate production is an important strategy used by *Lactobacillus* for promoting survival under acid stress. Conversion of lactate to 1, 2-propanediol by *L. buchneri* decreased the acidity of media. Further degradation of 1, 2-propanediol in *L. diolivorans* resulted in ATP production, which was critical for culture to survive acidic environmental after depletion of substrates during late stationary phase (Krooneman et al, 2002). Release of ammonia from both the conversion of glutamine to glutamate and glutamate to GABA increased the pH (Weingand-Ziadé et al, 2003; Richard and Foster, 2004). In addition, transportation of GABA also consumes extracellular protons and releases ATP (Higuchi et al, 1997; Richard and Foster, 2004). As strategies for acid resistance, both conversion systems are able to work under acidic conditions (Vermeulen et al, 2007; Ueno 2000; Park and Oh, 2007). Substrates for both conversion systems are abundant in sourdough. Lactate is the primary metabolite of both homofermentative and heterofermentative *Lactobacillus* strains, whereas glutamate and GABA production depends on availability of cereals used in fermentation. Wheat, rye and barley are rich in glutamine. During fermentation glutamine is continuously released, providing sufficient substrate for glutamate and GABA production in sourdough. Production of 1, 2- propanediol and propionate are less efficient than conversion of glutamine to glutamate or

glutamate to GABA. Glutamine was almost quantitatively converted to glutamate and then converted to GABA, whereas lactate is only partially converted to 1, 2-propanediol in *L. buchneri*, and conversion of 1, 2-propanediol to 1-propanol and propionate is at a ratio of around 1.5 to 1 in *L. diolivorans* (Krooneman et al, 2002). Compared to glutamate and GABA production, conversion of lactate to 1, 2- propanediol and conversion of 1, 2- propanediol to propionate are produced with comparable concentration. Up to 48 mM of propionate can be produced by cofermentation in *L. buchneri* and *L. diolivorans*, and rye malt sourdough with the addition of wheat gluten accumulated to around 90 mmol kg-1 DM GABA (Stromeck et al, 2011). Time required to produce propionate from lactate is much longer as it is primarily used for survival, whereas both conversion of glutamine to glutamate and glutamate to GABA are regular part of amino acids utilization. Besides GAD activity, glutamate can also be converted into α-KG by glutamate dehydrogenase (GDH) activity as reviewed by Liu et al, 2008. Alpha-KG a highly efficient amino group acceptor involved in flavour formation from amino acids, and was reported as the limiting factor for flavour production from amino acids in cheeses (Tanous et al, 2002; Yvon 2006). Alpha-KG is traditionally considered to be an amino group acceptor that is primarily responsible for further degradation of keto acids from amino acids to produce flavour compounds (Ardö 2006). Identification of α-KG as electron acceptor where conversion of α-KG leads to 2-OHG allows culture to regenerate NAD^+ and produce more acetate, which promoted culture survival as well.

Better understanding the metabolic potential of lactobacilli allows qualitative and quantitative production of desired products that contribute to nutrition, texture, or shelf life of sourdough bread. Identification of glutaminase activity in *L. reuteri* allows selection of a starter culture with highly specific glutaminase activity to produce glutamate, which possesses pleasant umami flavour and can be used to reduce the use of table salt (Yamaguchi and Ninomiya, 2000). Additionally, conversion of glutamine to glutamate allows further conversion of glutamate to GABA during sourdough fermentation, which is proven to possess antihypertensive property (Shimada et al, 2009). Production of propionate by cofermentation using *L. buchneri* and *L. diolivorans* promoted culture survival as well as extended shelf life of wheat sourdough bread.

Along with the rapid development of sequencing techniques, many genes encoding enzymes are available in various databases. Genome prediction of metabolic pathways is a powerful tool to assign hypothetical functions to unknown enzymes. Compared to traditional method of characterizing enzymes by individual strain selection and testing, using of genome prediction is much less time consuming. Screening of hundreds of strains at the same time becomes possible and results obtaining are no longer totally dependent on culture growth. However, industrial application relies on combination of genome prediction with biochemical analysis. Putative glutaminases have been identified in several *Lactobacillus* strains such as *L. johnsonii*; however, further confirmation and characterization of glutaminase were not carried out until this study (Pridmore et al, 2004). Conversion of α-KG to 2-OHG has been reported since Radler and

Bröhl (1984), and α-KG reductase activity has been reported in *E. coli.* However, since no putative α-KG reductase has been identified in *Lactobacillus* genomes, α-KG reductase has not yet been characterized.

Besides the combination of genomic and traditional biochemical technique to characterize the pathway, this study also emphasizes the use of novel application of known pathways in lactobacilli. *Lactobacillus* strains with same metabolic patterns from non-cereal fermentations can be applied to cereal fermentations. For example, cofermentation of *L. buchneri* and *L. diolivorans* produced propionic acid, where both two strains were originally used as silage inoculants. Additionally, increased industrial desire requires new understanding of known knowledge. Metabolism of α-KG has been reported since 1984, but use of this pathway in *L. sanfranciscensis* and *L. reuteri* during sourdough fermentation as electron acceptor to promote growth were not applied until this study. Moreover, glutaminase has been reported during sourdough fermentation for many years, and has been genetically characterized in pathogenic *E. coli*, but its functional characterization has only been concerned as the arrival of interest in wheat, rye and barley as those cereals are particularly rich in glutamine (Brown et al, 2008).

In conclusion, this thesis studied three metabolic pathways used by LAB during sourdough which contributes to bread quality, and offered characterization of those pathways at biochemical or genetic level.

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