Aroma relevant metabolic activities of lactobacilli during wheat sourdough fermentation

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Statements belonging to the dissertation of Nicoline Vermeulen

I. Eine holländische Doktorarbeit ist nicht vollständig ohne Thesen.

II. Intracellular redox homeostasis in sourdough microorganisms is extremely important for the over-all bread quality (*this thesis*).

III. The accumulation of amino nitrogen by *L. sanfranciscensis* in dough is attributable to peptide hydrolysis and gluten solubilization rather than proteolysis (*this thesis*).

IV. The amino acid metabolism by *L. sanfranciscensis* during growth in sourdough is limited by the peptide availability (*this thesis*).

V. In order to develop starter strains for specific applications, the amino acid metabolism of lactobacilli has to be studied on strain level. (*this thesis*).

VI. Lactobacilli can be used for *in situ* production of glutamate during food fermentation (*this thesis*).

VII. Der Verzicht auf Sauerteig in den Niederlanden und die Qualität holländischer Käse stehen in direktem Zusammenhang: Die Qualität der Käsesorten ist so gut, dass man von der Zugabe von Sauerteig, einem natürlichen Aromaspender und Geschmackverstärker, absehen kann (*diese Arbeit*).

VIII. Die Entwicklung wettbewerbsfähiger Produkte ist von neuen Erkenntnissen abhängig und daher ist die Grundlagenforschung, oft als „nicht praxis-relevant“ bezeichnet, zwingende Voraussetzung für Innovation.

IX. Brood, dáár zit wat in!

X. Der Mensch lebt nicht vom Brot allein (*Matthäus 4:4*)

Danksagung

Mein herzlicher Dank gilt meinem Doktorvater Prof. Rudi Vogel, für das mir entgegengebrachte Vertrauen und für das freundliche Arbeitsverhältnis.

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Teresa Zotta is acknowledged for the nice though short cooperation during her stay at Technische Mikrobiologie.

Erklärung

Ich versichere durch eigenhändige Unterschrift, daß ich die Arbeit selbstständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe.

Folgende Teile der Arbeit sind mit Hilfe von Dritten zu Stande gekommen:

- Die Teigfermentationen, die die Basis für Abbildung 9, 10 und 12 bilden, sind im Rahmen dieser Arbeit durchgeführt. Die in diesen Abbildungen dargestellten Ergebnisse beruhen auf gaschromatographischen Analysen, die von Herrn Dr. Michael Czerny an der Deutsche Forschungsanstalt für Lebensmittelchemie durchgeführt wurden. Der Methodenteil zu den Analysen (Paragraph 2.3.3) wurde freundlicherweise von Herrn Czerny zur Verfügung gestellt.

- Figur 16 basiert auf Daten, die der Diplomarbeit von Frau Hetty Machiltza entnommen wurden.

- Frau Claudia Thiele hat das fluoreszierende Casein zur Verfügung gestellt, das verwendet wurde für die in Abbildung 18 dargestellten Versuche.

- Die Abbildung 29 und 33 basieren auf Versuchen, die Frau Teresa Zotta (Erasmus-Austauschstudentin) unter meiner Betreuung am Lehstuhl für Technische Mikrobiologie durchgeführt hat.

Unterschrift

_____

Freising / Weihenstephan, 24. März 2006 Nicoline Vermeulen

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Abbreviations

AraT	aminotransferase, specific for aromatic amino acids
AU	arbitrary units
BcaT	aminotransferase, specific for branched-chain amino acids
bp	base pair
cbl	cystathionine-β-lyase
cDNA	copyDNA
cfu	colony forming units
cgl	cystathionine-γ-lyase
cxl	cystathionine lyase
cyst(e)ine	cysteine and / or its dimer cystine
DACM	N-(7-dimethylamino-4-methylcumarin-3-yl)-maleimid
Dpp	dipeptide permease
DtpT	Di- / tripeptide transporter
DTT	dithiothreitol
F	phenylalanine
FE	phenylalanyl-glutamate
FITC	fluorescein isothiocyanate
FL	phenylalanyl-leucine
FQ	phenylalanyl-glutamine
FS	phenylalanyl-serine
Gdh	Glutamate dehydrogenase
Glx	Glutamine / glutamate
GMP	Glutenin macropolymer
GSH	reduced glutathione (Glu-Cys-Gly)
GSSG	oxidized glutathione
HPLC	High-Performance Liquid Chromatography
Ldh	Lactate dehydrogenase
LP	leucyl-proline
LV	leucyl-valine
mMRS	Modified Man, Rogosa and Sharp medium
Opp	oligopeptide permease
ORF	open reading frame
PCR	Polymerase Chain Reaction
Pep	peptidase
PF	prolyl-phenylalanine
PLA	Phenyllactic acid

Prt	Extracellular serine proteinase
RP	Reversed-Phase
SDS	sodium dodecylsulfate
SH	sulfydryl
SS	disulfide bond
TD/HRGC/MS	Two-Dimensional High Resolution Gas Chromatography / Mass Spectrometry
TMW	Technische Mikrobiologie Weihenstephan

1 Introduction

Sourdough can be defined as 'a dough whose microorganisms originate from sourdough or a sourdough starter and are metabolically active or can be reactivated. Upon addition of flour and water they continue to produce acid' [1]. During sourdough fermentation, microorganisms (mainly yeasts and lactobacilli) grow and ferment sugars that are present in the dough. Lactic acid bacteria produce lactic and acetic acid as fermentation products, causing a drop in pH. Sourdough is of crucial importance for the production of rye breads. A gel consisting of pentosans and starch determines the volume of rye bread: during dough proofing, the gas produced by the microorganisms is trapped by a framework of these carbohydrates. At neutral pH, starch is degraded by α-amylase and consequently, gas originating from microbial metabolism cannot be retained within the loaf. At low pH however, α-amylase are inactive, gas remains within the bread structure and the result is an elastic bread crumb.

Sourdough fermentation is not essential for wheat bread production because wheat flour contains proteins that form a gas-retaining framework and carbohydrates play a secondary role only. Although it is not necessary, a substantial proportion of wheat bread is attained using sourdough fermentation [2;3]. Moreover, in yeasted preferments that are commonly used in wheat baking, lactobacilli usually grow to high cell counts when the fermentation time exceeds 8 – 16h [3;4]. The main motivation of the increasing use of sourdough and baking aids based on sourdough in wheat bread production are their beneficial effects on flavor [5;6;7], texture [8], microbial shelf life [9;10] and delayed staling [11]. Besides for rye and wheat bread production, sourdough fermentation is used for the Italian panettone, and can be used as quality enhancer for French baguettes, pizza and many other applications.

1.1 The aroma of sourdough

The term 'flavor' is generally used to refer to the over-all sensation that is perceived after food consumption. This includes taste and smell, but also mouth-feeling, color and sound. A clear distinction has to be made between taste and odor / aroma. Whereas the volatile compounds that reach the nose either directly or retronasal determine the aroma or odor of food, taste depends on the interaction of water-soluble compounds with the taste buds located on the tongue. There are 4 types of taste buds: for sweet, sour, bitter and salty. Flavor enhancers, e.g. glutamate, evoke a savory, delicious sensation called *umami*, which is Japanese for 'savory, delicious' when they are applied in concentrations above the perception threshold. Whether this is a fifth taste or not, is still under discussion. This sensation is not perceived when flavor enhancers are applied in low concentrations, but the over-all flavor is intensified. The savory flavor of protein hydrolysates like soy sauce can be explained by their high contents of free amino acids, especially glutamate [12]. For example, the addition of wheat during soy sauce fermentation could be explained by the fact that wheat is glutamine rich and therefore a suitable glutamate source.

The highly appreciated aroma of sourdough breads is caused by microbial activity during fermentation and does not rely on acidification only (for a recent review, see [13]). Microbially fermented sourdoughs were reported to contain higher levels of aroma compounds than chemically acidified doughs [14]. Levels of flavor volatiles in sourdough breads depend on the used sourdough starter [15;16;17;18;19]. The formation of volatile compounds during sourdough fermentation by lactic acid bacteria and yeasts differs significantly among and within the species [17]. Sensory panels have used different attributes to describe sourdough bread made using different starters [15;16].

Odorant levels in sourdough before and after fermentation with a commercial, mixed starter culture are shown in table 1. Acetic acid is a characteristic aroma compound in sourdough bread crumbs and is formed during sugar metabolism by heterofermentative lactobacilli. Further aroma compounds like 2- / 3-methylbutanoic acid and 2- / 3-methylbutanal are increased during sourdough fermentation (table 1). These aroma compounds in sourdough originate from microbial amino acid metabolism, and their formation will be discussed in detail in section 1.5.

Table 1. Changes in odorants during sourdough fermentation. Compounds that originate from lipid oxidation and amino acid metabolism are printed in bold and italics respectively [20].

	Amount in sourdough (µg)	
Compound	**Before fermentation** [a)]	**After fermentation** [b)]
3-methylbutanal	*51*	*158*
2-methylbutanal	*20*	*33*
2- and 3-methylbutanoic acid	*138*	*361*
3-(methylthio)propanal	*27*	*14*
hexanal	**2254**	**2070**
(Z)-4-heptanal	**4.1**	**0.8**
(E)-2-nonenal	**53**	**20**
(E,Z)-2,6-nonadienal	**13**	**3.8**
(E,Z)-2,4-decadienal	**363**	**52**
(E,E)-2,4-decadienal	**339**	**48**
acetic acid	49000	427000
butanoic acid	1510	4690
pentanoic acid	2440	4800
vanillin	592	288

[a)]: Estimated based on odorant levels in the sourdough starter and flour

[b)]: Amount determined after 24h of sourdough fermentation using an undefined, commercially available sourdough starter

Unsaturated aldehydes contribute to the crumb odor of wheat- and rye breads. (E,E)-2,4-decadienal (odor quality: deep fat fried) and (E)-2-nonenal (odor quality: fatty) are key odorants in wheat bread crumb [6;21] and contribute to crust aroma as well. (E,E)-2,4-decadienal and (E)-2-nonenal are generated by enzymatic oxidation of linoleic acid by endogenous flour lipoxygenases during the storage of flours and during dough mixing and fermentation. Baker's yeast as well as the sourdough microflora apparently have the ability to metabolize these important odorants: during sourdough fermentation started with a commercial starter preparation, the levels of these odorants in dough were

quantitatively reduced during 24h sourdough fermentation (table 1). A decline in unsaturated aldehyde levels was also observed during the proofing time of doughs fermented with baker's yeast [22]. The levels of (E)-2-nonenal and (E,E)-2,4-decadienal in sourdoughs were strain dependent [18;23] and the removal of (E)-2-nonenal by yeasts during dough fermentation depends on the fermentation conditions [24;25;26]. Since (E,E)-2,4-decadienal and (E)-2-nonenal are key aroma compounds in the wheat bread crumb, the fate of these compounds during fermentation will greatly determine the flavor of the bread. It is evident that the fermentation microflora of bread dough and sourdoughs determined the levels of these odorants in dough, but the contribution of the different microorganisms involved in the fermentation process – yeast, heterofermentative lactobacilli and homofermentative lactobacilli – remains unknown.

Whereas compounds generated during fermentation mainly determine the flavor of the bread crumb, thermal processes during baking are particularly important for odorant formation in the crust: potent odorants in the wheat bread crust originate from the chemical degradation of amino acids via the Maillard reaction or Strecker degradation. Sourdough fermentations with lactic acid bacteria result in an increase if amino acid concentrations during the fermentation [7;27], however, the levels of single amino acids released during fermentation are strain dependent [27]. For example, 2-acetyl-1-pyrrolin is a key odorant in wheat bread crusts [21] and originates from the Maillard reaction. Ornithine (a non-proteinaceous amino acid) is the precursor of 2-acetyl-1-pyrroline and can be formed by some lactobacilli, for example by *L. pontis* [28]. The roasty note of the wheat bread crust was enhanced using *L. pontis* as starter culture [7]. Besides the used starter culture, the sourdough bread aroma is affected by process parameters like fermentation time and the choice of wheat flour type [5;20] .

1.2 Technology and microbiology of sourdough

In traditional fermentations, sourdough is used as the sole leavening agent. However, in most industrial applications the combination of sourdough and baker's yeast as leavening agent is applied. A basic scheme of sourdough bread production is shown in figure 1. The sourdough bread aroma is affected by the choice of wheat flour type, process parameters like fermentation time and temperature, the use of baking aids and by the choice of starter culture [5;20]. Some dough ingredients like sucrose, malt and proteases increase the levels of reducing sugars and amino acids in the bread dough; these compounds are involved in thermal reactions during baking and the reaction products contribute to a dark-colored and well-smelling bread crust.

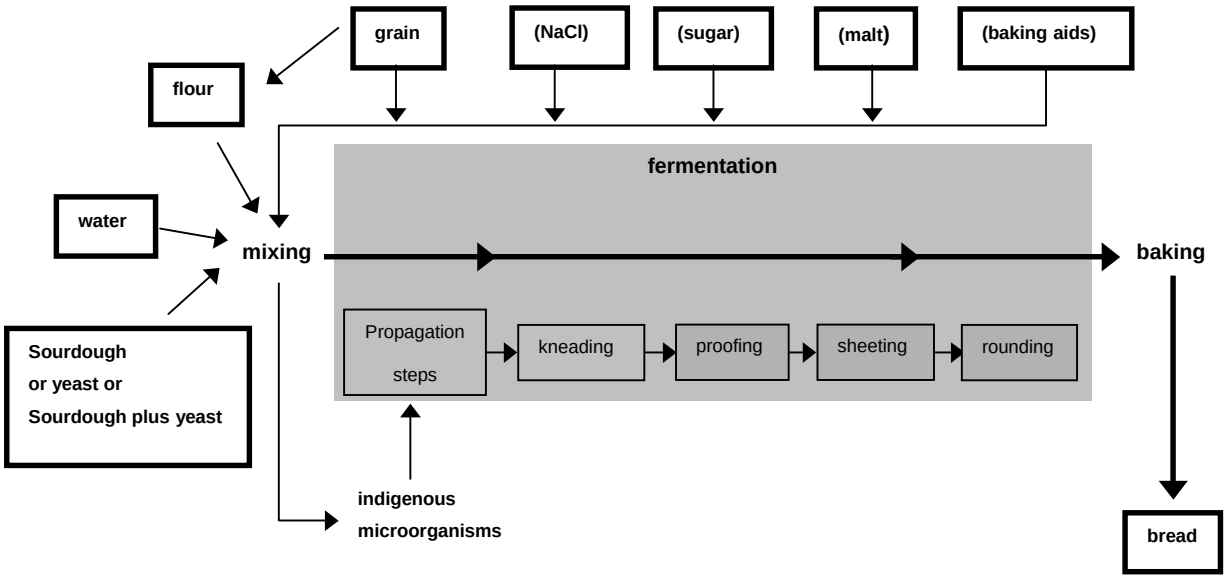

Figure 1. Basic scheme of dough fermentation for the production of baked goods [29].

During sourdough fermentation, the lactic acid bacteria use carbohydrates present in the flour for their energy supply, which results in the formation of acids and gas. The majority of lactic acid bacteria found in sourdoughs belongs to the genus *Lactobacillus* (*L.*) [29]. Lactobacilli can be divided into 3 groups, based on their central carbon metabolism: homo-, (obligatory) hetero- and facultative heterofermentative organisms. A schematic overview of these pathways is shown in figure 2. The homofermentative pathway follows the glycolysis (Embden-Meyerhof pathway), where 1 molecule hexose is converted to 2 molecules lactate, consuming 2 molecules ADP as cofactor and thus energy in form of 2 ATP-molecule is produced (pathway A). The heterofermentative lactic acid bacteria use the 6-phosphogluconate / phosphoketolase pathway (pathway B), forming 1 molecule each of lactate, ethanol, CO_2 and ATP from 1 molecule hexose. In the presence of external electron acceptors that can be used for NAD regeneration, e.g. fructose, citrate [30], heterofermentative lactic acid bacteria produce acetate instead of ethanol, which leads to additional ATP gain. Thus, the production of acetic acid (odor quality: pungent, vinegar) is influenced by the availability of electron acceptors (figure 2, [31]). Facultative heterofermentative organisms convert hexoses to lactate only, but in the presence of pentoses, phosphoketolase is activated and both lactic and ethanol or acetic acid are produced.

Most sourdough-related lactobacilli are heterofermentative organisms. The molar ratio between lactic and acetic acids produced during sourdough fermentation is called the fermentation quotient. As already mentioned, acetic acid influences the bread aroma and, from sensory point of view, the optimal value of the fermentation quotient is 2-2.7 [32]. It should be kept in mind that the fermentation quotient only reflect the proportion of the acids and that the absolute concentrations, which are important as well, are neglected.

Sourdough related lactic acid bacteria are adapted to the cereal environment by their ability to utilize maltose, the most common sugar in dough. The most dominant sourdough bacterium is *L.*

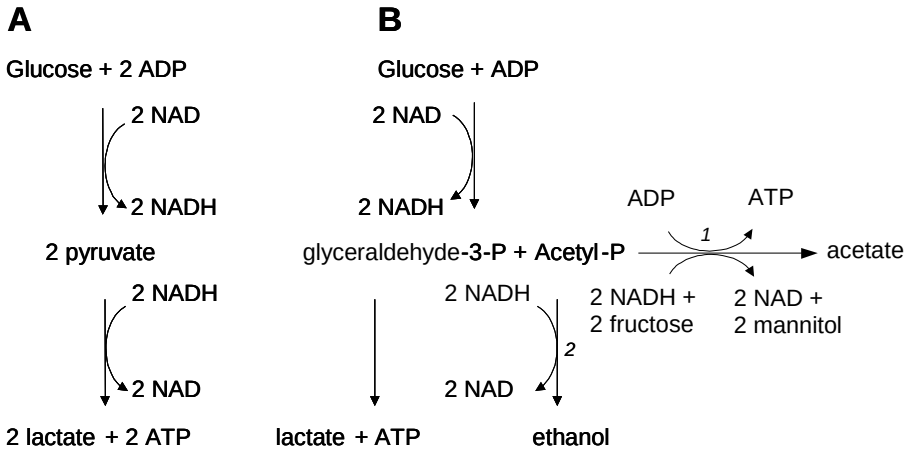

Figure 2. Schematic diagram of sugar catabolism by homofermentative and facultative heterofermentative (**A**) and obligative heterofermentative (**B**) lactobacilli. Metabolism via the Embden-Meyerhof-pathway (**A**) generates 2 ATP molecules per glucose molecule. Heterofermentative metabolism via the pentose-phosphate pathway generates 1 molecule of ATP per glucose molecule if acetyl-phosphate is used as electron acceptor to produce ethanol (*2*), or 2 ATP molecules in the presence of other electron acceptors, e.g. fructose, which enables additional ATP-synthesis through the conversion of acetyl-phosphate to acetate by acetate kinase (*1*).

sanfranciscensis and this bacterium has not been isolated elsewhere [29], other typical sourdough organisms are *L. pontis*, *L. mindensis*, *L. panis* and *L. frumenti* [2]. Ubiquitous species like *L. plantarum* and *L. brevis* are found in sourdoughs as well. Process parameters like fermentation time and temperature determine which bacteria dominate and therefore influence aroma formation, since the formation of odorants is depending on the metabolic features of the used starter culture. Basically, four different types of sourdough fermentations (type 0, I, II and III) are used in industrial practice [2;33]. Type 0 doughs are continuously propagated sponge doughs that are started with baker's yeast, but generally lactic acid bacteria develop to relevant cell counts in these doughs, and the final pH is decreased to 5.0 or less. The microflora of these doughs is dominated by baker's yeast and a large variety of homofermentative lactic acid bacteria, e.g. *L. plantarum* and *L. sakei*. Traditionally, sourdoughs are maintained by frequent inoculations at ambient temperatures (type I doughs), and breads obtained through traditional processes remain the high quality standard of bread in most European countries. Their microflora consists of yeasts and heterofermentative lactobacilli, mainly *L. sanfranciscensis*. Type II doughs are produced in a large-scale preparation at elevated temperatures for 2-5 days. In these doughs, low pH levels (pH < 3.5) are reached. Thermophilic, acid tolerant lactobacilli such as *L. pontis*, *L. panis*, *L. reuteri* and *L. frumenti* have been shown to dominate type II fermentations. Type III sourdoughs are started with defined starter cultures and are dried using spray- or drum drying. Consequently, only those organisms of the initial starter culture that resist drying can be isolated from these powders (e.g. *L. plantarum* or *L. brevis*) [2].

1.3 Diacetyl formation by lactic acid bacteria

Diacetyl (odor quality: buttery) is a by-product of pyruvate metabolism in a wide range of lactic acid bacteria, e.g. *Lactococcus lactis* and *L. delbrueckii* subsp. *bulgaricus*. It is a key aroma compound in dairy products like butter, yogurt and buttermilk. On the other hand, its production is undesirable in other products such as beer and wine.

The production of diacetyl by lactic acid bacteria has been extensively studied and is shown in figure 3. Many lactic acid bacteria harbor α-acetolactate synthase, which forms α-acetolactate from 2 pyruvate molecules. α-Acetolactate can undergo chemical oxidation resulting in diacetyl, or can be converted into acetoin by α-acetolactate decarboxylase (figure 3). Diacetyl is substrate for acetoin / diacetyl reductase, but acetoin is the preferred substrate and is a non-competitive inhibitor of the enzyme, which may explain the low reduction of diacetyl in environments with high amounts of acetoin [34].

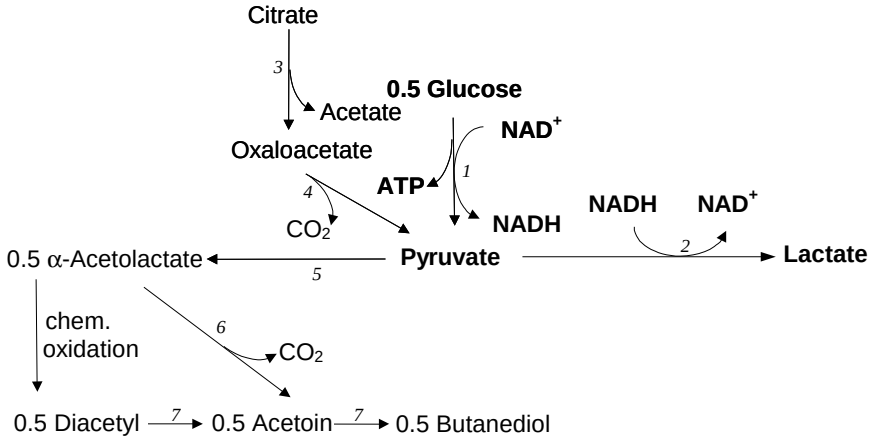

Figure 3. Diacetyl formation in homofermentative lactic acid bacteria. Main route for hexose degradation is printed in bold. α-Acetolactate is formed by α-acetolactate synthase from 2 molecules pyruvate, and α-acetolactate is chemically oxidized to diacetyl. Diacetyl-formation from pyruvate requires alternative substrates for cofactor regeneration such as citrate. Figure drawn according to [35]. *1*: glycolytic enzymes, *2*: lactate dehydrogenase, *3*: citrate lyase, *4*: oxaloacetate decarboxylase, *5*: α-acetolactate synthase, *6*: α-acetolactate decarboxylase, *7*: acetoin reductase.

α-Acetolactate can only be produced by α-acetolactate synthase if there is an intracellular excess of pyruvate, which mainly depends on the NAD / NADH balance. Homofermentative and facultative heterofermentative lactic acid bacteria convert 1 molecule glucose into 2 molecules pyruvate, which yields ATP but 'costs' NADH. NAD is regenerated by lactate dehydrogenase (figure 3). Many lactic acid bacteria are able to cometabolize citrate (figure 3), which results in additional pyruvate: pyruvate that is formed without the investment of NAD, and therefore, cofactor regeneration is not necessary and pyruvate becomes substrate for α-acetolactate synthase (figure 3). α-Acetolactate is a central metabolite, where catabolism and anabolism interfere; it is the precursor for acetoin / diacetyl, but it is also substrate in the biosynthesis of branched-chain amino acids. α-Acetolactate decarboxylase activity is controlled on gene expression level and on biochemical level by branched-chain amino acids [36]. Leucine is an allosteric activator of this enzyme: when leucine levels in the environment

are high, leucine biosynthesis is not needed and the metabolic flux of α-acetolactate is redirected form leucine towards acetoin production. Strains that lack α-acetolactate decarboxylase produce higher amounts of diacetyl compared to strains harboring the enzyme [37]. However, the reverse is not true: not all lactobacilli lacking α-acetolactate decarboxylase produce diacetyl [38]. In conclusion: diacetyl production is strain dependent and prerequisites are an intracellular pyruvate surplus and a low α-acetolactate decarboxylase activity.

In pastry and white wheat rolls, the consumer might consider a slight buttery odor pleasant. Furthermore, enhanced acetate levels in the bread might enhance the shelf life of the bread, since acetate inhibits fungal growth. During sourdough fermentation, manipulating the α-acetolactate decarboxylase activity cannot control the diacetyl production by lactobacilli, because the leucine concentrations cannot be artificially lowered; therefore, bacterial strains that display low α-acetolactate decarboxylase activity are needed for enhanced diacetyl production. Lowering lactate dehydrogenase activity and simultaneously increasing the α-acetolactate synthase activity was unsuccessful [39], probably because this has a severe impact on the redox balance. Aerobic conditions can cause pyruvate excess: NADH-oxidase positive strains can use oxygen as electron acceptor for NAD regeneration. Aeration enhanced diacetyl production by *L. lactis* [40;41], and it was speculated that an NADH oxidase overexpressing strain under aerated conditions produces even more diacetyl [41]. However, dough aeration is technically demanding and genetically modified organisms lack public acceptance. Citrate utilization may influence the bread aroma, because of the formation of acetate and diacetyl.

1.4 Wheat protein

The fate of the protein fraction of the flour during sourdough fermentation is of importance for bread quality for several reasons. First, the protein network in wheat doughs determines dough rheology and gas retention, and thus bread volume and texture. Furthermore, proteolytic events during fermentation provide the substrates for microbial conversion of amino acids to flavor precursor compounds and antifungal metabolites [7;9]. Third, some peptides from wheat proteins are involved in human cereal intolerance and their levels in bread hydrolysates are reduced by selected sourdough lactic acid bacteria [42].

Gluten proteins (for a recent review see [43]) are the main wheat flour proteins (85%) and can be divided into 2 fractions: the monomeric gliadins and the polymeric glutenins. The gliadins can be extracted from the flour using alcohol-water mixtures, whereas for the extraction of the polymeric glutenins the addition of a disulfide reducing agent is required. Gliadins have a molecular weight of 30.000-80.000 and can be divided in α-, β-, γ-, and ω-gliadins. After dough mixing, the proteins are linked to each other by disulfide, hydrogen and dityrosine [44] bonds, resulting in an insoluble network that is called the glutenin macropolymer (GMP) and can reach molecular masses up to several millions [45]. The polymerization of glutenins mainly depends on the formation of intermolecular

disulfide bonds. Low molecular weight compounds containing thiol (SH) groups, such as cysteine or the reduced tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH), can react with SH groups of the glutenins and gliadins and thus prevent oxidative cross-linking of glutenin proteins [46;47;48;49]. The quantity of the GMP in wheat flours determines their baking quality in straight dough processes.

During sourdough fermentation, a virtually quantitative depolymerization of the GMP is observed [50;51;52]. Sourdough addition to bread dough at a 20% level decreased the gluten quality in the resulting bread dough [50]. Acidification, either microbial or chemical, causes a degradation of the various protein fractions in sourdough, including the insoluble gliadins and glutenins [52;53]. Proteolytic enzymes in the flour are associated with wheat gluten. The pH-optimum of activity of these aspartic proteinases is below pH 4.0 [54;55]. Accordingly, the proteolytic activity in wheat doughs is strongly increased in acidified doughs independent of the presence of lactic acid bacteria [7;52].

The addition of reducing agents to wheat dough stimulates proteolytic activity, because the depolymerization of gluten proteins makes these more accessible for proteolytic degradation [7]. A virtually quantitative proteolytic degradation of gluten proteins to peptides and amino acids was observed in sourdough fermented with the heterofermentative *L. pontis* and an added fungal protease preparation [52]. In addition to proteolytic events, SS-SH interchange reactions catalyzed by lactobacilli may directly or indirectly interfere with gluten cross-linking via disulfide bonds and hence contribute to the depolymerization of the GMP.

It must be taken into account that fundamental differences exist between homofermentative and facultative heterofermentative lactobacilli on the one side, and heterofermentative lactobacilli on the other side with respect to their reductive capacity (figure 2). Some food-related facultative heterofermentative lactobacilli produce H_2O_2 during growth [56;57]. In contrast, heterofermentative species decrease the redox potential, because the efficient use of carbohydrates depends on the regeneration of reduced cofactor NAD(P)H. Heterofermentative lactobacilli from sourdough exhibit enzyme activities that generate low molecular weight SH compounds, like the glutathione reductase activity in strains of *L. sanfranciscensis* [58], and the cystathionine γ-lyase activity in several *Lactobacillus* species [59].

1.5 Amino acid metabolism in lactobacilli

Many lactic acid bacteria harbor an extracellular serine proteinase, Prt, which degrades protein to oligopeptides that are subsequently internalized and metabolized, which results in the formation of odor-active compounds. In table 1, it is shown that amino acid derived compounds play a significant role in the sourdough aroma. In sourdough, especially shortly after the fermentation is started, low molecular weight amino nitrogen is limited; therefore, solubilization of the GMP, proteolytic activity and transport efficiency are of crucial importance for the metabolite formation by lactobacilli and will be explained in section 1.5.1.

Metabolites from branched-chain and aromatic amino acids are particularly odor active. The metabolic pathways and regulation mechanisms involved in the conversion of these amino acids are very similar [60]. Thus, general interpretations may be derived from experiments with one of those amino acids. In this work, phenylalanine will be used as model amino acid, since phenyl groups are easy to detect and the metabolites of phenylalanine are both odor active and antifungal, its conversion by lactic acid bacteria will be introduced in section 1.5.2. The metabolism of sulfur-containing amino acid follows different pathways and will be discussed in section 1.5.3. Furthermore, special attention will be given to the conversion of glutamine by lactobacilli in section 1.5.4, because wheat protein is extraordinary rich in glutamine [43],.

1.5.1 Proteolytic activity of lactobacilli

The proteolytic system of lactic acid bacteria is best studied in *Lactococcus lactis* (for reviews see [61;62;63]). Although less extensively studied, the main features of the proteolytic system of lactobacilli appear to be similar to that one of *L. lactis*. It employs an extracellular serine proteinase, Prt, which degrades protein to oligopeptides that are subsequently internalized and metabolized. The Prt proteinase has been characterized on genetical and biochemical level in several species of lactobacilli [63], although not all lactobacilli are *prt*-positive. This enzyme is a serine proteinase, calcium dependent, located in the cell-envelope and its pH-optimum is approximately pH 5.5-6.5 [64]. Proteolytic activity is of vital importance for lactic acid bacteria in milk products like yogurt or cheese, since the levels of essential amino acids are too low to support growth [64;65]. When peptide levels are high in the environment, the *prt* expression of lactic acid bacteria is lowered [66;67]. In meat, initial protein degradation originates from muscle enzymes (so-called cathepsins), but the addition of proteolytic starter cultures influence the further degradation of the protein fragments thereby enhancing the sensorial attributes of fermented sausage [68;69]. Proteolytic activity in sourdough extracts is inhibited by aspartic protease inhibitors but not by serine protease inhibitors [51]. Thus, it seems that the most active proteases in wheat sourdoughs are the cereal aspartic proteinases, whereas serine proteinases from lactobacilli play a minor role only [7]. On the other hand, it has been shown that lactobacilli display enzyme activities that contribute to protein degradation during dough fermentation [70;71]. Furthermore, a serine proteinase has been isolated from the cell envelope of *L. sanfranciscensis* CB1 and was shown to be able to hydrolyze gliadins [71].

Lactic acid bacteria meet their nitrogen requirements mainly by oligopeptide transport followed by peptide hydrolysis [72;73;74;75]. It is energetically more favorable to first transport peptides and then hydrolyze them intracellular than transporting every single amino acid one by one. Hence, it seems likely that peptides are more efficiently internalized and converted into metabolites than amino acids. However, all published studies on amino acid metabolism by lactobacilli have employed amino acids rather than peptides as substrates and the possibility to increase amino acid turnover by an optimized supply of substrates remains to be elucidated.

Three different peptide transport systems were identified in *L. lactis*; Opp, DtpT and Dpp. DtpT and Dpp are single proteins, and transport hydrophilic di- and tripeptides. Opp is an ATP dependent system that consists of 5 proteins, which mainly transports oligopeptides [76]. Peptidases hydrolyze the peptides to single amino acids that thereafter are further metabolized. The Opp transport system was genetically characterized in *L. delbrueckii* [77], peptidases corresponding to enzymes from *L. lactis* are widely distributed amongst lactobacilli [61]. From *L. sanfranciscensis* CB1, a dipeptidase and an aminopeptidase have been purified and characterized [71]. Preliminary evidence indicates that lactobacilli use peptides during growth in sourdough to meet their substrate requirements [42;52].

1.5.2 Phenylalanine conversion by lactobacilli

Starting from the intracellular pool of amino acids, amino acid metabolism in lactic acid bacteria (for a recent review see [60]) is initiated by a transamination reaction [78;79;80] where the α-amino group is transferred to a keto acid acceptor by an aminotransferase. In lactococci, α-ketoglutarate is the main amino acceptor [80;81], which is then converted into its corresponding amino acid; glutamate. Other α-keto acids such as pyruvate and oxaloacetate may serve as amino acceptors as well [82]. After removal of the amino group, the resulting α-keto acid can be metabolized by several enzymatic reactions, resulting in the corresponding aldehyde or alcohol, carboxylic acid or hydroxy acid. The proportion of the metabolites formed is strain dependent [79;83].

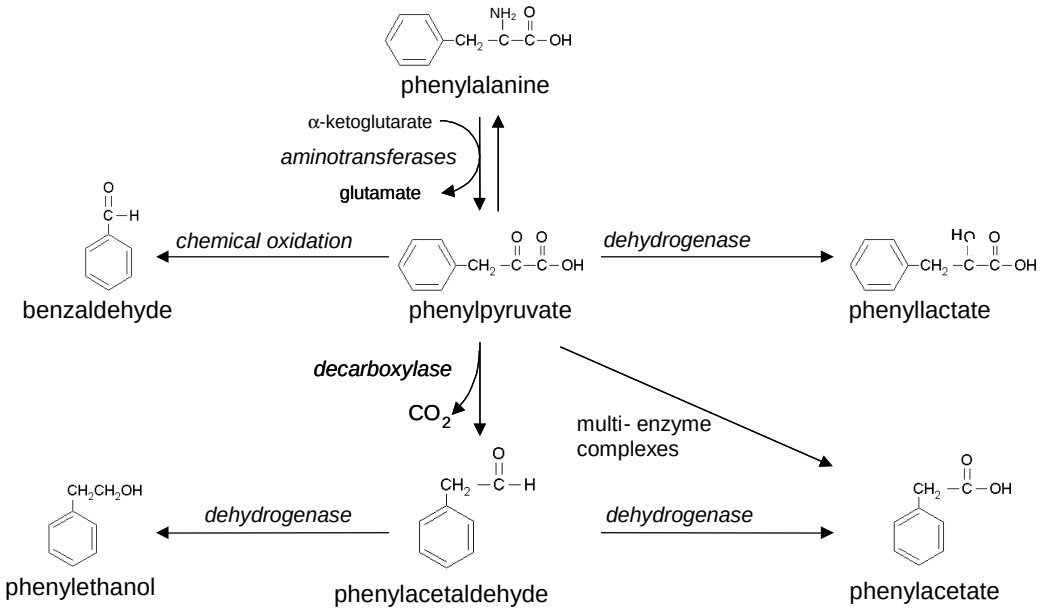

Figure 4. Pathways involved in phenylalanine metabolism in lactic acid bacteria. After [84], modified.

A schematic overview of phenylalanine degradation by lactic acid bacteria is given in figure 4. Phenylpyruvate is generated after transamination and can then be reduced by hydroxy acid dehydrogenases [78;85], which results in the production of phenyllactic acid (PLA). When phenylpyruvate is decarboxylated, phenylacetaldehyde is formed, which in turn can be converted to

phenylethanol or phenylacetate. When phenylpyruvate is not enzymatically converted, it may undergo a chemical oxidation to benzaldehyde [86].

In lactic acid bacteria, amino acid metabolism is limited by the availability of amino acceptors in the transamination reaction. It has been shown that the addition of α-ketoglutarate [87;88;89] or pyruvate [90] during cheese making enhances the amino acid degradation. The conversion of amino acids was increased by using a glutamate dehydrogenase (Gdh) overproducing strain, that was able to produce extra α-ketoglutarate from glutamate under the reduction of NAD to NADH [91]. Tanous et al. [92] have shown a positive correlation between the Gdh activity in lactic acid bacteria and their ability to metabolize amino acids. Cometabolism of citrate and glutamate resulted in higher amino acid conversion rates [93].

In *L. lactis*, some of the genes encoding enzymes involved in phenylalanine metabolism were identified. The expression of the peptide transporter *opp*, of several peptidases and of the aminotransferases *araT* and *bcaT* are under CodY repression [62;94]. CodY senses the intracellular pool of branched-chain amino acids [95 ;96]. Branched-chain amino acids stimulate CodY's binding to the DNA, which leads to inhibition of the gene transcription and thus down-regulates peptide transport and hydrolysis as well as the amino acid conversion. It is not known to which extent the regulation described for *Lactococcus* is valid for sourdough lactobacilli, nor how the formation of the intracellular amino acid pool, the transamination, the conversion of phenylpyruvate and the regulation of these processes influence the formation of PLA during sourdough fermentation.

Many metabolic pathways are linked to co-factor availability, which is influenced by central carbon metabolism. Previous studies on amino acid metabolism and co-factor regeneration have focused on homofermentative lactobacilli. Under conditions prevailing in sourdough, *L. plantarum* uses the Embden-Meyerhof-pathway for sugar fermentation whereas *L. sanfranciscensis* is obligatory heterofermentative as most major representatives of the microflora in traditional sourdoughs. In this group of organisms, the NADH dependent enzymatic reactions are strongly influenced by citrate and fructose. These co-substrates enable cofactor regeneration [30] and thus potentially increase amino acid turnover via an increased Gdh activity.

PLA, one of the catabolites from phenylalanine, inhibits microbial growth. Bread spoilage is mainly due to the growth of filamentous fungi, and metabolites produced by lactobacilli can inhibit their growth. In addition to lactic and acetic acid, minor metabolites originating from amino acid conversion display antifungal activity [97]. PLA is one of these minor metabolites is. Mold growth was delayed by 5 days in wheat bread produced using sourdough started with a PLA producing *L. plantarum* strain in comparison to a bread produced using sourdough started with a non-PLA producing *L. brevis* strain [9]. PLA is produced by a wide range of *Lactobacillus* species, but its production is strain dependent [98;99;100]; PLA was formed by lactic acid bacteria growing in MRS broth in levels up to 99 mg L^{-1} [100].

1.5.3 Metabolism of sulfur-containing amino acids by lactobacilli

The sulfur containing amino acids cysteine, its dimer cystine (together abbreviated cyst(e)ine) and methionine and their metabolites may influence the bread quality in different ways: (i) Sulfuric compounds of low molecular weight are highly odor-active and may be generated in sourdough fermentation through bacterial metabolism of these amino acids; (ii) Thiol containing compounds interfere with the gluten network and may thus affect the dough rheology and bread texture. The enzymes involved in the metabolism of methionine and cysteine are well characterized in dairy lactococci (for a review see [101]). Methionine can be substrate for the branched-chain and aromatic aminotransferases discussed in section 1.5.2 [80;102], but there are specific pathways for the metabolism of sulfur-containing amino acids as well. A schematic overview of these pathways is depicted in figure 5.

Both lactococci and lactobacilli contain high levels of enzymes that act on cystathionine [103].

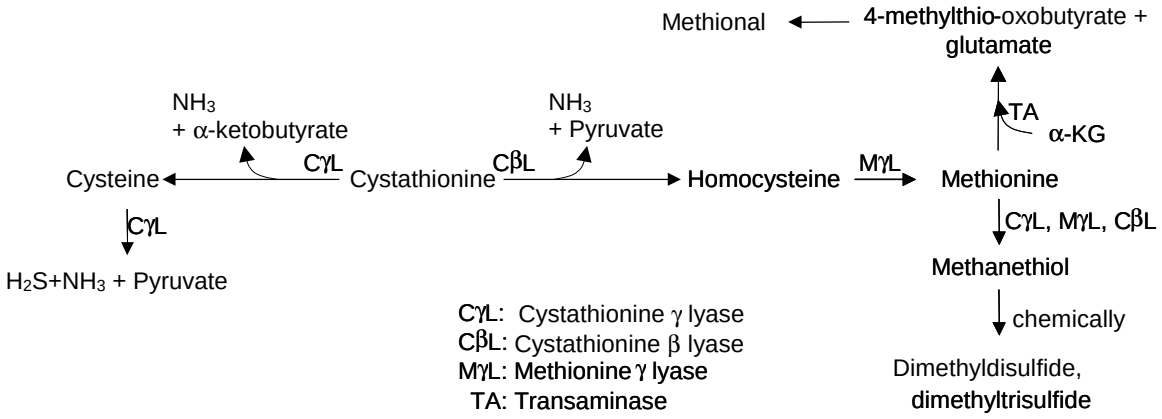

Figure 5. Metabolism of sulfur-containing amino acids in lactobacilli. α-KG: a-ketoglutarate. After [101], modified

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Cystathionine lyase activity has been demonstrated in several lactic acid bacteria, including *Lactococcus lactis* [104;105], *L. fermentum* [106], *L. delbrueckii* subsp. *bulgaricus* [107] and *L. reuteri* [108]. This pyridoxal-5'-phosphate dependent enzyme is involved in the cysteine biosynthesis and, on account of its broad substrate specificity, is able to degrade methionine and cysteine to methanethiol and hydrogen sulfide, respectively. Cystathionine lyases catalyze either α,β-elimination (cystathionine-β-lyase, EC 4.4.1.8) or α,γ-elimination (cystathionine-γ-lyase, EC 4.4.1.1) or both eliminations. Since the characterized cystathionine lyases are active under cheese ripening conditions and low molecular weight substances play a large role in cheese flavor, the importance of this enzyme activity seems obvious [104;105;106]. The use of starter cultures with high cystathionine lyase activities during cheese ripening correlated with higher levels of methanethiol, dimethyldisulfide and dimethyltrisulfide as compared to cheeses started with starter cultures with low cystathionine lyase activities [108]. Seefeldt and Weimer [109] showed that lactobacilli can produce volatile sulfur-containing compounds and that this ability is strain dependent. Methional (odor-quality cooked potato like) plays an important role in both sourdough [18;20] and bread aroma [6;21]. However, little

information is available on the metabolism of sulfur containing amino acids in cereal associated lactic acid bacteria and its influence on bread quality.

1.5.4 Glutamine deamidation by lactobacilli

Flour protein is rich in glutamine; approximately 40% of the amino acid residues in gluten are glutamine [110]. Due to this high glutamine content of flour protein and the proteolytic activity of the flour, glutamine should be present in abundance in sourdough. Glutamine deamidation by lactobacilli during sourdough fermentation could therefore cause an increase in glutamate levels. Since this is a flavor enhancer, this metabolic activity of lactobacilli can be expected to influence bread flavor. Lactobacilli could profit from glutamine deamidation in several ways: (i) One of the reaction products, ammonium, is a basic compound and could therefore help the cell to avoid acidic stress as it occurs during a prolonged sourdough; (ii) Yeasts prefer NH_4^+ as nitrogen source over amino acids [111], the production of NH_4^+ by lactobacilli from glutamine could decrease the competition for amino acids during a cofermentation.

In literature, few indications can be found that lactobacilli indeed display glutamine deamidation activity. Crude cell free extract of *Lactococcus lactis* subsp. *cremoris* B78 displayed deaminase activity on glutamine, asparagine and arginine [104]. During a buffer fermentation, some strains seemed to cause a rise in glutamate levels, whereas other strains did not [112]. *Lactobacillus casei* and *L. paracasei* subsp. *paracasei* caused a decrease in glutamine levels and an increase in glutamate levels [113], indicating glutaminase activity. In dry fermented sausages started with a mixed culture, a rise in ammonium concentrations was observed upon the addition of free amino acids [114]. In cheese, the same effect was observed [115]. Taken together, these observations indicate that starter cultures actively deamidate glutamine, giving rise to glutamate and ammonium. This assumption was confirmed by Weingand-Ziade et al. [116], who characterized glutaminase activity in broken cells from *L. rhamnosus*. Furthermore, a sharp increase in glutamate and aspartate during sourdough fermentation has been observed [117]. This could be explained by glutamine deamidation, because wheat protein is low in aspartate, asparagine, and glutamate.

1.6 Metabolic activities of lactobacilli and their possible influence on wheat sourdough aroma

The superior aroma of sourdough breads is caused by the microbial activity during fermentation and does not rely on acidification only (for a recent review, see [13]). The development of odorants during sourdough as presented in table 1, points out that lactobacilli are likely to contribute to the bread aroma in several ways. Besides strain dependent features, like the formation of diacetyl, more general metabolic pathways of lactobacilli might influence the levels of odorants and odorant precursors in sourdough and the corresponding bread. It should be mentioned that aroma relevant metabolic activities of lactobacilli will often interfere with other important dough or bread properties. For example, extensive proteolysis gives rise to amino acids, but causes a collapse of the GMP as well.

Furthermore, the conversion of sulfur containing amino acids will probably influence thiol levels, which in turn influence gluten cross-linking.

The degradation of proteins during dough contributes to the aroma formation in two ways: amino acids are precursors for thermal reactions that greatly influence the crust aroma and they can be metabolized by lactobacilli giving rise to specific metabolites. Metabolites from branched-chain, aromatic and sulfur containing amino acids are known to be particularly odor-active and consequently, their formation during cheese ripening has been thoroughly studied (for reviews see [60] for branched-chain aromatic amino acids and [101] for sulfur-containing amino acids). These metabolites could be accumulated during sourdough fermentation as well, and could influence the crumb aroma. Furthermore, the conversion of glutamine into glutamate could greatly influence bread flavor, since glutamine levels are extraordinary high in wheat protein. Furthermore, it is evident that the fermentation microflora of bread dough and sourdoughs determined the levels compounds originating from lipid oxidation in dough (table 1), but the contribution of the different microorganisms involved in the fermentation process – yeast, heterofermentative lactobacilli and homofermentative lactobacilli – remains unknown.

1.7 Aim of this thesis

The over-all aim of the work was to screen for and to characterize metabolic activities of lactobacilli that are relevant for the sourdough and bread aroma, because the superior aroma of sourdough is caused by the microbial activity during fermentation and does not rely on acidification only. More knowledge of the aroma relevant metabolic pathways and their regulation will be useful for product development. The physiological performance of a wide variety of lactobacilli during sourdough fermentation was studied in order to screen for interesting metabolic properties. Diacetyl formation during sourdough formation was studied, since in pastry and white wheat rolls, the consumer might consider a slight buttery odor pleasant. Levels of unsaturated aldehydes originating from lipid oxidation change during dough fermentation (table 1). The fate of (E)-2-nonenal and (E,E)-2,4-decadienal during sourdough fermentation and the underlying metabolic pathways were studied.

Metabolites from amino acid fermentation are important odorants in sourdough (table 1). Flour is low in amino acids, but their levels rise during fermentation. Dough proteolysis was studied from two different perspectives: (i) the influence of lactobacilli on gluten cross-linking was investigated and (ii) *L. sanfranciscensis* DSM20451^T was screened for proteolytic activity.

The formation of an intracellular amino acid pool was studied in *L. sanfranciscensis* DSM 20451^T and the subsequent conversion of amino acids into low molecular weight compounds was studied in several *Lactobacillus* species. Although the factors limiting amino acid metabolism in *L. lactis* are increasingly understood, the current knowledge does not enable the optimization of phenylalanine turnover by sourdough lactobacilli. It was the objective to determine the role of peptide supply and co-substrates as rate limiting factors in metabolite formation from phenylalanine in *L. plantarum*

TMW1.468 and *L. sanfranciscensis* DSM20451^T. The influence of the substrate, the cofactor availability and the presence of branched-chain amino acids on PLA formation were studied, as well as the phenylpyruvate dehydrogenase and glutamate dehydrogenase activities.

Besides aromatic amino acids, metabolites from sulfur containing amino acids and glutamine could also play a role in aroma formation during sourdough fermentation. Thus, it was decided to investigate the degradation of cysteine in preferments and the distribution of cystathionine lyase amongst lactic acid bacteria for use in sourdough fermentation. Furthermore, from earlier reported data (section 1.5.4), one can deduct that it is likely that lactobacilli convert glutamine into the taste enhancer glutamate during dough fermentation. Therefore, it was investigated whether lactobacilli are able to deamidate glutamine and glutaminyl –residues and whether glutamate levels in doughs were increased by lactobacilli.

2 Materials and Methods

2.1 General methods

2.1.1 Strains and culture conditions

The microorganisms used in this work were chosen from the Technische Mikrobiologie Weihenstephan (TWM) strain collection. Strains isolated from sourdoughs, cereal and non-cereal fermentations were included, and the selection covered homofermentative, facultative heterofermentative and obligatory heterofermentative organisms. The selected strains are shown in table 2. Modified Man, Rogosa and Sharp medium (mMRS) contained per L: 5 g glucose x H_2O, 5 g fructose, 10 g maltose, 10 g peptone from casein, 5 g meat extract, 5 g yeast extract, 4.0 g KH_2PO_4, 2.6 g K_2HPO_4 x $3H_2O$, 3.0 g NH_4Cl, 1 mL Tween 80, 0.1 g $MgSO_4$ x $7H_2O$, 0.05 g $MnSO_4$ x H_2O, 0.5 g L-Cys HCl x H_2O, 0.2 mg each of biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamin, riboflavin, cobalamin and panthothenic acid; and 15 g agar for solid media. The pH was adjusted to 6.2.

Table 2. Lactic acid bacteria used for strain selection

Species, strain #, origin			Fermentation type / metabolites	Species, strain #, origin			Fermentation type / metabolites
L. sanfran..	1.52	SD	heterof.	*L. reuteri*	1.106	SD	heterof., orn, glucan, GOS, fructan, FOS, reutericyclin, colonization of mice
	1.53	SD	heterof., fructan, FOS, isogenic with DSM20451^T		1.976	duck	heterof., glucan, GOS
	1.392	SD	heterof. fructan, FOS	*L. frumenti*	1.666	SD	heterof., fructan, FOS
	1.949	SD	heterof.		1.103	SD	heterof., fructan, FOS
	1.1149	SD	heterof.	*L. fermentum*	1.890	SD	heterof.
	1.727	SD	heterof.	*L. sakei*	1.22	meat	homof.,
L. perolens	1.500	beer	homof., diacetyl	*L. panis*	1.648	SD	heterof.
	1.501	beer	homof., diacetyl	*L. brevis*	1.465	Beer	heterof.,
L. pontis	1.397	SD	heterof., orn	*L. amylolyticus*	1.1078	SW	homof.
L. plantarum	1.460	beer	homof.	*L. mucosae*	1.81	SD	heterof.
	1.468	beer	homof.	*W. confusa*	1.327	beer	heterof.
L. mindensis	1.1206	SD	homof.		1.928	SD	heterof.

#: TMW strain number, *L. sanfran*: *L. sanfranciscensis,* SD: sourdough, beer: beer spoilage organism, duck: duck intestinal isolate, meat: starter culture for meat fermentations, SW: sour wort, homof., heterof.: homo- and heterofermentative strains, respectively, GOS, FOS: formation of glucose and fructose-oligosaccharides from sucrose, respectively, orn: formation of ornithine from arginine.

2.1.2 Sourdough fermentations

If not stated otherwise, white wheat flour type 550 (German classification; type 550 wheat flour has an ash content of 0.51-0.63%) was obtained at a local supermarket and was used for sourdough fermentation. Doughs were inoculated with single lactobacilli (initial cell count approximately 10^7 colony forming units / g dough) or yeast (initial cell count approximately 10^5 colony forming units / g dough) strains. To inoculate sourdoughs, overnight cultures (volume: 20% of the final dough weight) were washed twice with tap water. Doughs were thoroughly mixed by hand and incubated at 30ºC. The absence of contaminations was verified by observation of a uniform colony morphology on agar plates. Dough samples were frozen at -20ºC.

2.1.3 Determination of cell counts and pH

The dough pH and the cell counts were determined as described previously [53]. In chemically acidified doughs, cell counts remained generally below 10^4 colony forming units (cfu) / g. In sourdoughs, the strains grew to high cell counts (approx. 1.0×10^9 cfu / g) after 24h of fermentation, and the dough pH was <4.0 after 24h of fermentation. The absence of contaminants in sourdoughs was verified by observation of a uniform colony morphology.

2.1.4 Determination of carbohydrates and organic acids

The concentrations of maltose, glucose, and fructose as well as the metabolites lactate, acetate, ethanol, phenyllactic acid, phenylethanol, phenylacetate and phenylacetaldehyde were determined by high-performance liquid chromatography. Sourdough samples were diluted 1:1 with distilled water; medium samples were used undiluted. After overnight protein precipitation using 3.5% perchloric acid, the precipitates were removed by centrifugation (15000 x g, 20 min). The 20 µL samples were eluted from a Polyspher^R OA CK column (300 x 7.8 mm, Merck, Darmstadt, 70°C) with 5 mM H_2SO_4 in 5% acetonitrile at a flow of 0.4 mL min^{-1}. Phenyl group containing metabolites, citrate and α-ketoglutarate were detected using a UV detector set at 210 nm. All other compounds were detected using a refractive index detector.

α-Acetolactate is very instable and can therefore not be purchased. A 10µM solution of α-methyl-α-acetoxyethyl acetoacetate (purchased from Sigma Aldrich, Germany) was flushed with nitrogen, and mixed 1 to 1 with a 10 µM NaOH solution under anaerobic conditions. The reaction mixture was injected on the HPLC system described above. The reaction was incomplete, HCOOH, acetic acid, ethanol, α-acetolactate and a residue of α-methyl-α-acetoxyethyl acetoacetate were present in the solution. Thus, the retention time of α-acetolactate could be determined, but not it's response factor.

2.1.5 Determination of single amino acids and total amino nitrogen

For amino acid analysis, samples were prepared and analyzed according to Thiele et al. [118]. Total amino nitrogen was determined using the modified ninhydrin method described by Thiele et al. [7]. Dough samples were clarified with perchloric acid as described in section 2.1.4. Clear supernatant (100 µL) was mixed with 20 µL 3 M KCl to precipitate the perchloric acid. After 1 h at room temperature, the precipitate was removed by centrifugation (10 min at 15000 x g). Reagent 1 (50 µL, 5.0 g Na_2HPO_4 x 2 H_2O, 6.0 g KH_2PO_4, 0.5 g ninhydrin and 0.3 g fructose in 100 mL H_2O, pH 6.7) was added to 5 µL sample and 95 µl H_2O, the reaction mixture was incubated at 100 °C for 16 minutes at. The samples the reaction was stopped by cooling on ice, subsequently, 30 µL of sample was transferred into a microtiterplate, the wells of which already contained 50 µL reagent 2 (0.2 g KIO_3 solved in 60 ml deionized water and 40 ml ethanol). The absorbance was measured at 590 nm in a microtiterplate spectrophotometer (TECAN spectrafluor, Grödig, Austria). A calibration curve was prepared with each measurement using glycine as standard and results were expressed as mmol glycine L^{-1}. The standard deviation of the assay was generally less than 5 %.

2.1.6 General molecular techniques

Cloning, DNA manipulations and agarose gel electrophoreses were done as described by Sambrook et al. [119]. Chromosomal DNA was isolated from *L. sanfranciscensis* using an E.Z.N.A. Bacterial DNA kit (Peqlab Biotechnologie GmbH, Erlangen, Germany). PCR reactions were carried out with Taq-polymerase from Qbiogene (Montréal, Canada). PCR products were separated by gel electrophoresis, bands of the expected size were isolated from the gel using an E.Z.N.A Gel Extraction kit (Peqlab Biotechnologie GmbH) and sequenced by Sequiserve (Vaterstetten, Germany). Enzymes and reagents were used according to the manufacturers recommendations unless otherwise stated.

2.2 Influence of citrate addition on the sourdough and bread aroma

2.2.1 Dough fermentation and bread making

Cells from 100 mL overnight *L. perolens* TMW1.501 culture were washed two times with tap water, resuspended in 10 mL tap water. Of this cell suspension, 5 mL was added to 250 g sourdough, dough yield 200. For one sourdough, citrate was dissolved in the tap water, the pH was adjusted to 6.5 and the solution was mixed with 125 g white wheat flour and cell suspension. The other dough was prepared from 125 g wheat flour (type 550), 125 g tap water and cell suspension. Doughs were fermented for 24 h at 30°C. After fermentation, the dough pH was 3.5 or 4.0 in the citrate-free or citrate-containing dough, respectively. The absence of contaminants in sourdoughs was verified by observation of a uniform colony morphology. Fermented sourdoughs were used for sensory analysis directly or for bread baking. Bread doughs (dough yield 160) were prepared using white wheat flour (750 g), tap water (405 g), fresh, compressed yeast (15 g) and sourdough or preferment (225 g). The

doughs were kneaded for 3 minutes, proofed for 60 minutes and baked at 220°C for 45 minutes. Before sensory analysis, the bread was allowed to cool down for 1 hour.

2.2.2 Sensory analysis of sourdough and bread

Triangular tests were performed with an untrained panel consisting of 10 untrained TMW-employees (5 men / 5 women). The samples (24h fermented *L. perolens* sourdough, containing 50 mM citrate or the corresponding bread, respectively) were presented in triangular tests in parallel with two samples of reference sourdoughs 24h fermented *L. perolens* sourdough, without citrate or the corresponding bread, respectively). Ca. 20 mL sourdough or a little piece of bread crumb was filled in 50 mL glass beakers that were covered with Parafilm. The panelists were asked to mark the sample differing from the two others in the overall aroma. The number of panelists that were able to discern the differing sample from the reference samples was determined and the significance of the result was statistically checked according to Jellinek [120]. Furthermore, the panelists were asked to describe the aroma of both the reference and the sample.

2.3 Reduction of (E)-2-nonenal and (E,E)-2,4-decadienal during sourdough fermentation

2.3.1 Dough fermentations

Whole meal wheat flour and white wheat flour type 550 (ash content of 0.51-0.63%) was obtained at a local supermarket. Dough formulas are given in table 3. Doughs were inoculated with single strains of lactobacilli or yeast strains to obtain cell counts of approximately 10^7 and 10^5 colony forming units (cfu) / g, respectively. To inoculate sourdoughs, overnight cultures were washed twice with tap water. Doughs were thoroughly mixed and incubated at 30ºC. Samples were taken and frozen at -20ºC. Cell counts were determined during fermentation to verify the growth of the starter strains and the absence of contaminations was verified by observation of a uniform colony morphology on agar plates. Samples from doughs with a dough yield of 200 (A1-A5) were taken and frozen at -20ºC. Samples from doughs with a dough yield of 400 were shock frozen in liquid nitrogen (dough B1-C4, E1) or in an -80ºC ethanol bath (dough D1-D3).

Table 3. Dough formulas for preparation of sourdoughs and chemically acidified doughs

Dough	Fermentation time	Starter culture	Organic acids	Flour (g)	Tap Water (g)	(E,E)-2,4-Decandienal	(E)-2-Nonenal
A1	96h	*L. sakei*	-	1000	1000	-	-
A2	96h	*L. sanfranciscensis*	-	1000	1000	-	-
A3	96h	*L. reuteri*	-	1000	1000	-	-
A4	96h	None	13.2 mL [a)]	1000	1000	-	-
A5	48h	*S. cerevisiae*	13.2 mL [a)]	1000	1000	-	-
B1	320 min	*L. sakei*	-	100	300	4 mg / mL EtOH	-
B2	320 min	*L. sanfranciscensis*	-	100	300	4 mg / mL EtOH	-
B3	320 min	None	-	100	300	4 mg / mL EtOH	-
B4	320 min	*S. cerevisiae*	-	100	300	4 mg / mL EtOH	-
C1	320 min	*L. sakei*	-	100	300	-	4 mg / mL EtOH
C2	320 min	*L. sanfranciscensis*	-	100	300	-	4 mg / mL EtOH
C3	320 min	None	-	100	300	-	4 mg / mL EtOH
C4	320 min	*S. cerevisiae*	-	100	300	-	4 mg / mL EtOH
D1	320 min	*L. sanfranciscensis*	-	100	300	4 mg / mL EtOH	-
D2	320 min	*S. cerevisiae*	-	100	300	4 mg / mL EtOH	-
D3	320 min	None	-	100	300	4 mg / mL EtOH	

[a)]: Mixture of 10 volumes lactic acid (90%) to 1 volume acetic acid (100%)

2.3.2 Hexanal conversion by *L. sanfranciscensis* and it's influence of on glucose metabolism

Overnight cell cultures of (50 mL) of *L. sanfranciscensis* TMW1.52 were harvested by centrifugation, washed twice with imidazole buffer (50 mM, pH 5.5) and resuspended in 2.5 mL imidazole buffer. To 750 µL imidazole buffer containing 5 g L^{-1} maltose and 2% hexanal stock solution (125 mM hexanal in ethanol), 250 µL cell suspension was added to achieve a final hexanal concentration of 1.9 mM. Fermentation buffer containing 2% ethanol without hexanal was used as a substrate blank. The samples were incubated by 30°C for 0, 2 and 6 hours. Fermentations were stopped by the addition of 50 µL 70% perchloric acid. After protein precipitation, solids were removed by centrifugation and the samples were injected two times on a high-performance liquid chromatography (HPLC) system equipped with a Polyspher^R OA KC column (300 x 7.8 mm, Merck, Darmstadt, 70°C). Organic acids were quantified by elution of 20µL samples with 5 mM H_2SO_4 at a flow of 0.4 mL min^{-1} and detected using a UV detector set at 210 nm. Hexanal and hexanol were quantified by elution of 20 µL samples with 5 mM H_2SO_4 in 5% acetonitrile at a flow of 0.4 mL min^{-1} and detected using a refractive index detector.

2.3.3 Quantification of (E,E)-2,4-decadienal, (E)-2-nonenal and their corresponding alcohols and saturated aldehydes

2.3.3.1 Chemicals

Reference compounds nos. 1, 2, 4, and 5 (table 4) were obtained from Sigma-Aldrich (Steinheim, Germany). The following isotopic labeled standards were synthesized according to the literature given in parenthesis: [$^2H_2$]-1 [121], [$^2H_{3-5}$]-2 and [$^2H_{1-2}$]-4 [122], [$^2H_{3-5}$]-5 [121].

Table 4. Selected m/z-Ions and Calibration Factors used in Stable Isotope Dilution Assays

no.	compound	selected ion of analyte (m/z)	internal standard[a)]	selected ion of int. std. (m/z)	calibration factor[b)]
1	(E)-2-nonenal	141	[$^2H_2$]-1	143	0.72
2	nonanal	143	[$^2H_{3-5}$]-2	146-148[c)]	0.97
3	(E)-2-nonenol	69	[$^2H_{1-2}$]-3	70-71[c)]	0.70
4	nonanol	71	[$^2H_{1-2}$]-4	72-73[c)]	0.63
5	(E,E)-2,4-decadienal	153	[$^2H_{3-5}$]-5	156-158[c)]	1.04
6	(E,E)-2,4-decadienol	137	[$^2H_{3-5}$]-6	140-142[c)]	0.92

[a)]: Isotopic labelling of the internal standard
[b)] :Calibration factors obtained by analyzing a 1+1 mixture (w/w) of the analyte and the internal standard.
[c)] :The sum of the relative abundances of the ions was calculated.

2.3.3.2 Syntheses

(E,E)-2,4-decadienol (6): a solution of (E,E)-2,4-decadienal (1.52 g, 10 mmol) dissolved in anhydrous diethyl ether (20 mL) was slowly dropped to a suspension of $LiAlH_4$ (10 mmol, 0.40 g) in anhydrous diethyl ether (20 mL), stirred for 30 min at room temperature and refluxed for additional 30 min. After

cooling to room temperature, aqua dest. (10 mL) and hydrochloric acid solution (1 mol / L, 5 mL) were slowly dropped to the synthesis. The organic phase was separated and the aqueous phase was extracted with diethyl ether (3 x 50 mL). The organic phases were combined, dried over anhydrous Na_2SO_4, filtrated, and concentrated to about 1-2 mL by distilling off the solvent on a Vigreux-column (40 x 1 cm). Aqua dest. (5 %, m / m) was homogenisated with silica gel 60 (Aldrich, Steinheim) which was purified according to Esterbauer [123] by shaking for 1 h. A water-cooled column (30 x 2 cm, 12°C) was packed with a slurry of the silica gel 60 in n-pentane. After application of the synthesis (2 mL), the silica gel was rinsed with the following n-pentane / diethyl ether mixtures (v / v): fraction A 95 / 5 (75 mL), fraction B 90 / 10 (75 mL), fraction C 80 / 10 (75 mL), fraction D 50 / 50 (75 mL), and fraction E 0 / 100 (100 mL). The fractions were analyzed by GC / MS and the target compound was detected in fraction D.

MS (EI): 83 (100), 41 (99), 67 (77), 79 (75), 55 (73), 84 (72), 81 (69), 70 (45), 69 (39), 80 (31), 77 (28), 54 (28), 68 (23), 39 (22), 97 (22), 53 (19), 154 (18, M^+), 82 (18), 98 (16), 93 (15), 91 (14), 65 (14), 136 (14), 110 (13), 66 (13), 95 (13), 43 (11), 78 (11).

MS (CI): .137 (100), 81 (96), 67 867), 95 (63), 83 (47), 84 (37), 69 825), 79 823), 70 (21), 136 (20), 68 819), 94 (14), 98 (14), 80 (13), 82 812), 65 (12), 138 (11), 97 (11).

(E)-2-nonenol (3): The synthesis was carried out according to the synthesis of (E,E)-2,4-decadienal using (E)-2-nonenal (1,40 g, 10 mmol) instead of (E,E)-2,4-decadienal.

MS (EI): .57 (100), 41 (43), 55 (37), 43 (30), 68 (24), 82 (24), 67 (23), 54 (21), 81 (20), 95 (19), 56 (19), 69 (18), 96 (14), 70 (12), 39 (11), 71 (10).

MS (CI): .69 (100), 83 (52), 125 (9).

[$^2H_{3-5}$]-(E,E)-2,4-decadienol ([$^2H_{3-5}$]-6): The synthesis was carried out according to the synthesis of (E,E)-2,4-decadienol using a solution of [$^2H_{3-5}$]-(E,E)-2,4-decadienal (about 2 µmol, 3.0 mg) dissolved in diethyl ether (5 mL) and a suspension of $LiAlH_4$ (5 mmol, 0.20 g) in anhydrous diethyl ether (10 mL) instead of the reported chemicals without purification by column chromatography.

MS (EI): .83 (100), 85 (81), 81 (70), 41 (68), 84 (68), 79 (66), 70 (62), 80 (57), 55 (55), 69 (43), 56 (43), 67 (42), 68 (37), 57 (34), 82 (26), 71 (26), 42 (25), 78 (23), 77 (19), 97 (19), 95 (18), 99 (18), 54 (18), 66 (17), 86 (17), 53 (16), 98 (16), 72 (15), 96 (14), 39 (12), 93 (12), 94 (11), 65 (10), 58 (10), 100 (10).

MS (CI): .83 (100), 141 (97), 84 (80), 142 (69), 85 (58), 98 (50), 68 848), 67 (44), 82 (44), 97 (44), 70 (43), 69 (41), 140 (41), 99 (34), 71 826), 96 (21), 81 (19), 79 816), 153 (15), 65 (14), 95 (12), 72 811), 86 (11), 80 (10).

[$^2H_2$]-(E)-2-nonenol ([$^2H_2$]-3): The synthesis was carried out according to the synthesis of (E,E)-2,4-decadienol using a solution of [$^2H_2$]-(E)-2-nonenal (about 2 µmol, 2.8 mg) dissolved in diethyl ether (5 mL) and a suspension of $LiAlH_4$ (5 mmol, 0.20 g) in anhydrous diethyl ether (10 mL) instead of the reported chemicals without purification by column chromatography.

MS (EI): .59 (100), 56 (54), 55 (43), 41 (39), 69 (38), 70 (35), 43 (35), 58 (31), 83 (30), 57 (29), 42 (28), 97 (28), 84 (25), 71 (21), 68 (19), 82 (17), 96 (14), 72 (11), 85 (10).
MS (CI): . 70 (100), 69 (64), 84 (49), 71 (40), 85 (37), 83 (18), 75 (10), 127 (10).

2.3.3.3 Quantification of compounds nos. 1-6

Wheat dough (20 g) was mixed with dichloromethane (10 mL) (m / v) and the formed emulsion was spiked with known amounts of isotopic labeled standards. Low amounts of dough (1 g) were diluted with tap water (20 mL). After stirring for 30 min, the emulsion was distilled in high vacuum using SAFE-distillation technique [124]. The organic phase of the distillate was separated in a separating funnel and the aqueous phase was extracted with dichloromethane (3 x 20 mL). The organic phases were combined and dried over anhydrous Na_2SO_4. After filtration, the extract was concentrated to about 0.1 mL at a Vigreux column (40 x 1 cm) and subsequently by microdistillation [125]. The concentrated extract was analyzed by TD / HRGC / MS.

2.3.3.4 Two-Dimensional High Resolution Gas Chromatography / Mass Spectrometry (TD/HRGC/MS)

TD/HRGC/MS was performed using the moving column stream switching (MCSS) system (ThermoQuest Analytical Systems, Egelsbach, Germany) as described by [126]. Mass chromatograms were recorded in the MS/CI-mode using methanol as reagent gas.

2.3.4 Influence of redox-reactions catalyzed by homo- and heterofermentative lactobacilli on gluten in wheat sourdoughs

2.3.5 Sourdough fermentations

Two flours were used for sourdough fermentations. Wheat flour obtained at a local supermarket (ash content of 0.55%) was used to determine the effect of fermentation on amino acid- and sulfhydryl levels in sourdoughs. Flour prepared from wheat of the cultivar Batis (protein content 11.2%, ash content 0.567%) was kindly provided by the Bayerische Landesanstalt für Landwirtschaft, Freising, Germany and used for the determination of SH levels in gluten proteins.

To inoculate sourdoughs, cells from 10 mL overnight culture were washed two times with tap water, resuspended in 1 mL tap water and used to inoculate 50 g sourdough to a level of $1 \pm 0.5\ 10^7$ colony forming units (cfu) / g. Sourdoughs with a dough yield of 200 were mixed by hand and incubated at 30°C. Chemically acidified doughs were prepared using a 1:4 acetic acid / lactic acid mixture, in order to mimic the acidification by lactobacilli, 3.75 µL / g dough were added after 2 hours of fermentation to adjust the pH to 4.5, and 6.25 µL were added after 7 hours to adjust the pH to 3.6. GSH was added to a concentration of 10 µmol / g dough as indicated.

2.3.6 Measurement of free thiol groups in sodium dodecyl sulfate (SDS)-soluble protein fractions

Dough was extracted with 50 mM sodium phosphate buffer (pH 6.9) containing 1.5% SDS at a 1:10 extraction ratio. Otherwise, extraction of dough was performed according to Thiele et al. [53].

The concentration of free thiol groups in dough was determined essentially according to Antes and Wieser [127]. 75 µL SDS-soluble extract was mixed with 150 µL reagent A (n-propanol (50%) in 50 mM sodium phosphate buffer, pH 8.0, saturated with nitrogen gas in order to avoid oxidation of the SH groups) and 7.5 µL reagent B (39.6 mg 5,5-dithiobis-(2-nitrobenzoic acid) in 10 mL 0.5 M sodium phosphate buffer, pH 7.0). After the incubation for 30 min in the dark, the absorbance was measured at 405 nm. GSH solutions with concentrations ranging from 0 to 0.42 mM were used for the calibration curve. Data are reported as means of duplicate independent experiments and the experimental error was generally less than 10%.

2.3.7 Sequential extraction of wheat protein

Extraction of wheat proteins from dough was performed essentially according to Wieser et al. [128]. Dough samples were extracted twice with each solvent. The dough:solvent ratio was 1:2 during the first extraction step, and 1:1 during the second extraction step. Extraction was performed at room temperature, initiated by vortexing and followed by over-head shaking for 30 minutes. Insoluble material was removed from the suspension by centrifugation (10000g, 5 min). The following solvents were used: Solvent I (0.4 M NaCl, 150 mM NaH_2PO_4, 150 mM Na_2HPO_4, solvent II (50% v / v 2-propanol), solvent III (50% v / v 2-propanol, 1% acetic acid, 1% dithiothreitol). In solvent I, a higher sodium phosphate concentration was used as described by Wieser et al. (1998) in order to compensate for the acidity of the sourdoughs.

2.3.8 Labeling of free thiol groups in gliadin fractions and reversed-phase (RP) high-performance liquid chromatography (HPLC)

The labeling of free thiol groups in the gliadin fraction was performed essentially according to Antes and Wieser [127]. The extracts were incubated at 80°C for 2 minutes in order to inactivate present enzymes. To 1 mL extract, 0.5 mL solvent A (50% v / v 2-propanol in 50 mM sodium phosphate buffer, pH 8.0), and 0.5 mL solvent B (10 mL acetone containing 0.15 mg DACM (Molecular Probes Europe, Leiden, Netherlands)) were added and the reaction mixture was incubated at 37°C in the dark for 20h.

The protein content in unlabeled gliadin fractions and the distribution and amount of thiol groups in the labeled gliadin fractions was measured using an RP-HPLC method based on the method of Wieser et al. (1998). A C18 RP column (250 x 4.6 mm, 300 Å, 10 µm particle size, Phenomenex, Torrence, USA) was eluted at 70° using the following solvents: A: 0.1% trifluoroacetic acid in water and B 0.1% trifluoroacetic acid in acetonitrile. The flow rate was 1 mL / min, the following gradient was used: 0 to

2 min: 24% B, 2 – 32 min: gradient from 24% to 56% B. UV absorbance was measured at 210 nm, the fluorescence detector (Gynktotek RF 1002 Fluorescence Monitor, Germering, Germany) was set to an excitation wavelength of 378 nm and an emission wave length of 480 nm. Data are representative for quadruplicate independent experiments.

2.3.9 Glutathione reductase activity of *L. sanfranciscensis*

Cells of *L. sanfranciscensis* TMW1.53 grown over night were harvested by centrifugation, washed twice with imidazole buffer (200 mM, pH 5.5) and resuspended in imidazole buffer with 10% of the initial volume. To 450 µL imidazole buffer containing 13 mM oxidized glutathione (GSSG), 150 µL cell suspension was added to achieve a final GSSG concentration of 10 mM. The samples were incubated by 30°C for 2 and 6 hours. Fermentations were stopped by the addition of 30 µL 70% perchloric acid. After protein precipitation, solids were removed by centrifugation and the samples were injected on a HPLC system equipped with a RP C18 column (Luna, 250 x 4.60 mm, 5 µm particle size, Phenomenex, Torrence, U.S.A.) coupled to a UV detector set to 210 nm. The flow was 1 mL / min, eluents A and B consisted of 0.1% trifluoroacetic acid in water and acetonitrile, respectively. Samples were eluted with the following solvent gradient: 0 to 5 min: 2% B, 5 to 25 min: gradient from 2 to 20% B, followed by elution with 100% B as a column wash, and re-equilibration to 2% B.

2.3.10 Assessment of glutathione reductase (*gshR*) expression in dough

The oligonucleotides, based on the gene sequence of glutathione reductase of *L. sanfranciscensis* TMW1.53 (accession number AJ874718), were as follows: GshRfor: 5'-gggagtggacatggaacg-3', GshR-rev: 5'-gcttcaggcaatagttgaatc-3'. Genomic DNA and copyDNA synthesized based on mRNA isolated from sourdough served as templates in polymerase chain reaction (PCR). PCR and agarose gel electrophoresis were performed as described by Sambrook et al. [119].

2.4 Proteolytic activity of *L. sanfranciscensis* DSM20451^T

2.4.1 Screening the *L. sanfranciscensis* genome for *prt*

Degenerated primers were based on conserved *prt* sequences of *L. lactis* and lactobacilli (table 5). The primers were targeted on six distinct highly conserved regions, taking into account the codon usage in the various *prt*-genes of lactic acid bacteria.

Table 5. Primers used for PCR detection of *prt*.

	Primer sequence [a)]	Target AA sequence [b)]
PrtF4[c)]	GTTGCYTCYGCTGARAAC	VASAEN
PrtF5	TCTGCYGGKAACTCMGG	SAGNSG
PrtF6	GGKGCYGAYGTYMTMAACATG	GADV(L/I)NM
PrtR5	CCRAARAAGCCCTRTATGG	PYMGFFG
PrtR6	AAATGGHGARGCCATTGAMGT	TSMASPF
PrtR7	CCTGGWGCDGWRATWTCTGG	P(D/E)I(T/S)APG

[a)]: For mixed base sites, the code of the International Union of Biochemistry is used
[b)]: Consensus target amino acid sequence based on the alignment of the cell-wall associated serine proteinases PrtP of *L. lactis* (J04962), PrtB of *L. delbrueckii* (AAC41529.1), PrtP of *L. paracasei* subsp. *paracasei* NCDO 151 (M83946.1), PrtH of *L. helveticus* (AF133727), and a cell wall-associated serine proteinase of *L. johnsonii* NCC 533 (NC_005362).
[c)]: PrtF4, 5, and 6, forward primers, PrtR5, 6, and 7, reverse primers

2.4.2 Sourdough fermentations

Wheat flour with an ash content of 0.55% was obtained at a local supermarket. To inoculate preferments, cells from 50 mL overnight culture were washed two times with tap water and resuspended in 5 mL tap water. Preferments were made of 25 g flour, 20 g tap water and 5 mL cell suspension, stirred with a spatula and incubated at 30°C for 16 hours. Preferments were used to inoculate the sourdoughs that were prepared using the dough formulas shown in table 6. Sourdoughs were incubated at 30°C and samples were taken after 0 h, after fermentation to a dough pH of 4.8 (ca. 5 hours), and after 24 hours, corresponding to a pH of 3.6. In order to inhibit aspartic proteinases, pepstatin A (Sigma-Aldrich) was added to a final concentration of 40 µM from a stock solution in methanol:acetate 9:1 prepared daily.

Table 6. Dough formulas for preparation of sourdoughs and chemically acidified doughs

	Sourdough with *L. sanfranciscensis*			Chemically acidified dough		Sourdough with *L. sanfranciscensis*		Chemically acidified dough	
	SF	SF-Pe	SF-A	C	C-A	SF	SF-A	C	C-A
additives	none	peptone	pepstatin A	none	pepstatin A	none	pepstatin A	none	pepstatin A
flour (g)	135	135	9	5	5	0.755	0.755	0.800	0.800
tap water (g)	135	135	9	5	4.95	0.755	0.755	0.800	0.800
preferment (g)	30	30	2	-	-	0.09	0.09	-	-
peptone (g)	-	3	-	-	-	-	-	-	-
lactic acid (µL)[a)]	-	-	-	192	192	-	-	17.3	17.3
acetic acid (µL)[a)]	-	-	-	48	38	-	-	4.3	4.3
FITC-casein (µL)	-	-	-	-	-	100	100	100	100
pepstatin A (mg)	-	-	0.54[b)]	-	0.27[b)]	-	0.002	-	0.002

[a)]: 40% of the volume indicated was added after 2h, taking into account acetic acid added with the pepstatin A-stock solution, the remaining volume was added after 7h.
[b)]: pepstatin A was added to achieve a final concentration of 40 µM, stock solutions of pepstatin A in methanol:acetic acid [9:1] with a concentration 5.4, 2.7, and 2.2 mg mL^{-1} were used for doughs SF-A, C-A, and C-A with fluorescent casein, respectively

2.4.3 Determination of proteolytic activity of sourdoughs

The proteolytic activity of sourdough systems was investigated by monitoring the hydrolysis of fluorescent casein prepared according to Twining et al. [129]. The use of fluorescein isothiocyanate (FITC) labeled casein was preferred over the use of FITC-gliadin or –glutenin to avoid problems associated with gluten solubility in dough and laboratory media [52;130]. Dough formulas are shown in table 6. The doughs were incubated at 30°C for 5 and 24 hours. To 200 mg sourdough, 300 µL distilled water and 20 µL 70% perchloric acid were added in order to precipitate proteins. After overnight precipitation, solids were removed by centrifugation. Supernatants were neutralized by the addition of 4 volumes Tris-HCl buffer (pH 8, 1 M). In order to determine fluorescence-labeled hydrolysis products of fluorescent casein, the fluorescein fluorescence intensity was measured in a microtiterplate spectrophotometer (TECAN spectrafluor, Grödig, Austria).

2.4.4 Total RNA isolation from sourdough and copyDNA (cDNA) synthesis

One hundred grams sourdough were suspended in 200 mL Tris-HCl buffer (50 mM, pH 7.0) and centrifuged to remove large particles (5 minutes, 1500 g). Cells of *L. sanfranciscensis* predominantly remained in the supernatant and were harvested by centrifugation (15 min, 4500 g) and resuspended in 3 mL Tris-HCl buffer (50 mM, pH 7.0) with 3 mL RNAprotect (Qiagen, Hilden, Germany). This cell suspension was used for RNA isolation with the Qiagen RNeasy Mini kit. DNA was removed by treating the sample with RQ1 RNase-free DNase (Promega). Reverse transcription was performed by incubation of RNA with random hexamer primers (Random hexadeoxynucleotides) at 70°C for 10 minutes. After cooling on ice, 1 µL dNTPs (25 mM), 1µL reverse transcriptase (200 U μL^{-1}, M-MLV-RT, RNase H minus, Promega), 5µL reaction buffer (supplied with reverse transcriptase) and 5 µL

RNase free water were added. The sample was incubated at 25°C for 10 minutes, subsequently at 42°C for 110 minutes and heating the sample at 72°C for 15 minutes stopped the reaction.

2.4.5 Real-Time PCR

Real-time PCR was performed in a LightCycler Instrument (Roche Molecular Biochemicals, Mannheim, Germany). The primers used for real-time PCR are shown in Table 7. The reaction mix consisted of 10 µL QuantiTect SYBR Green PCR Master Mix, (Qiagen), 2 µl cDNA, 6 µl DNase free water, 1 µL of each primer (final concentration 0.5 pM). The PCR reaction was carried out in glass capillaries. Crossing points (CP, the point at which the fluorescence rises appreciably above the background fluorescence [131] were determined performing the 'Fit Point Method' in Roche LightCycler Software 3.5. In order to determine the real-time PCR efficiency, standard curves using diluted chromosomal DNA were included for each primer pair. The efficiency (E) was calculated from the slopes given by the LightCycler Software according to the equation: $E = 10^{(-1/\text{slope})}$ [131]. In cDNA libraries, the amount of the respective target genes was calculated relative to their amount in the control, i.e. the cDNA libraries prepared from cells exponentially growing in sourdough without additives. To account for variation in the mRNA isolation and cDNA synthesis, differences in the amounts of target genes were normalized against the differences in the *ldh* according to the following equation [131]:

$$N = \frac{E_{target}^{(CP_{target,control} - CP_{target,sample})}}{E_{reference}^{(CP_{reference,control} - CP_{reference,sample})}}$$

E_{target} and $E_{reference}$ represent the efficiencies of the respective PCR reactions, target genes were *oppF*, *dtpT* and *pepT*. L-*ldh* encoding for the lactate dehydrogenase was used as reference gene in order to relate the transcription of the target genes to the central carbon metabolism *Ldh* was preferred as reference over ribosomal rRNAs to avoid the comparison of stable, high copy number rRNA with fast degradable, low copy number mRNA [131]. The efficiencies of the PCR reactions were determined as 1.61, 1.78, 1.89 and 2.15 for *oppF*, *dtpT*, *pepT* and *ldh*, respectively.

Table 7. Oligonucleotide pairs used for determining gene expression of *L. sanfranciscensis* DSM20451^T.

Target gene	**Oligonucleotide sequences (Forward / Reverse)**	**Size PCR product (bp)**
pepT	5'-CCAATATTTGATTGCTCACCC-3'	219
	5'-GGATGCACGTTGGTTCC-3'	
ldh	5'-TACTGGTCGTTTCCGTG-3'	280
	5'-CGTTCTCATTAAAGCAGA-3'	
oppF	5'-GGTTCATCCGCAATTACC-3'	171
	5'-CCTTACTCCTGAACAGG-3'	
dtpT	5'-CTTCGTCGCGAAAGTGCG-3'	227
	5'-CCTTCTCCATCAAGCAAAGG-3'	
pepC	5'-GTCCACTGTGCTTTTGATTG-3'	145
	5'-TCGGAACATAAAATTGTTGC-3'	
pepX	5'-AAGAATTGAAAACCGAAAGC-3'	154
	5'-GAATTGTCACCACACTCGTC-3'	
pepR	5'-GGTTGATCGGATCGGTGACTAC-3'	215
	5'-GAGCAATGGCAGCAGGTTTC-3'	
oppA-pepN	5'-CGTGTTAAGGGTTACAGTG-3'	256
	5'-CATAAAAACGTTCAATTTTAGCC-3'	

2.4.6 Accession numbers

Sequences are submitted at EMBL Nucleotide Sequence Submissions (http://www.ebi.ac.uk) and have the following accession numbers: *dtpT*: AJ866920, *oppF*: AJ866923; *oppD*: AJ866924; *oppA-pepN*: AJ866928; *pepX*: AJ866927, *pepR*: AJ866925, *pepT*: AJ866926; *pepC*: AJ866922, *ldh*: AJ866921.

2.5 Phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM20451^T and *Lactobacillus plantarum* TMW1.468

2.5.1 Fermentation conditions

After 2 sequential overnight fermentations, precultures were used to inoculate 1.2x concentrated N-limited mMRS at 2.5%. In N-limited mMRS, peptone and meat extract were omitted and 5 g L^{-1} yeast extract was added as sole source of complex nitrogen. Stock solutions of peptides (phenylalanyl-leucine, FL; phenylalanyl-serine FS; phenylalanyl-glutamate, FE; prolyl-phenylalanine, PF; leucyl-valine, LV; leucyl-proline, LP; all obtained from Bachem, Weil am Rhein, Germany), amino acid, α-ketoglutarate, citrate and fructose, or, when necessary, water were added in order to dilute the medium to 1.0x concentrations. The pH-value of the stock solutions was adjusted to pH 6. Fermentations were allowed to proceed for 72 h at 30ºC. Samples were taken after 0, 6, 24 and 72 h and immediately frozen.

2.5.2 Sourdough fermentations

Wheat flour with an ash content of 0.55% was obtained at a local supermarket. To inoculate sourdoughs, 5 mL of an overnight culture were washed twice with tap water. The pellet was resuspended in 5 mL tap water and this cell suspension was mixed with 10 g flour and 5 mL tap water

containing the required additives (F, FS, citrate, α-ketoglutarate, fructose, 5 mM, pH 7). Sourdoughs were incubated at 30ºC and samples were taken after 0, 6, 24 and 48 hours.

2.5.3 Concentrations of phenylalanine and phenylalanine-containing peptides in fermentation samples

For determination of phenylalanine and phenylalanine-containing peptides, sample preparation was the same as used for the detection of sugars and organic acids. The 20 µL samples were injected on a C18 RP column (250 x 4.6 mm, 300 Å, 5 µm particle size, Phenomenex, Torrence, U.S.A.) coupled to a UV detector set to 210 nm and 257 nm, the flow was 1 mL min^{-1}. Solvents A and B consisted of 0.1% trifluoroacetic acid in water and acetonitrile, respectively. Before injection, the column was equilibrated with 2% B for 5 minutes. Samples were eluted with the following solvent gradient: 0-25 min: gradient from 2% to 20% B, 25 – 40 min: gradient from 80% to 100% B.

2.5.4 Measurement of glutamate dehydrogenase activity in cell free extract

Glutamate dehydrogenase activity was measured essentially according to Tanous et al. [92]. Cell free extract was prepared from 50 mL overnight grown cells. The cells were washed twice with reaction buffer (50 mM MOPS, pH 9), cell pellet was resuspended in 5 mL reaction buffer and cells were lysed using ultrasonication. Cell debris was removed using centrifugation (6000 g, 10 min). The reaction mixture for the measurement of the Gdh activity consisted of 5mM glutamate and 5 mM NADH or NADPH. The oxidation of NAD(P)H was measured by measuring the absorbance of the reaction mixture at 340 nm. As a control, cell-free extract was inactivated at 90ºC for 5 minutes. Substrate-free reaction mixture was included as well. Protein concentration in the cell free extracts was measured using the BioRad Protein Assay (BioRad Laboratories GmbH, Munich, Germany) according to the manufacturers directions. Bovine serum albumin was used for the calibration curve.

2.5.5 Amplification of *codY*

Degenerated primers targeting *codY* were designed based on conserved *codY* sequences of *L. lactis* subsp. *lactis* (AE006253) of *L. delbrueckii* subsp. *lactis* (AX118803).

The following primer pair was used (for mixed base sites, the code of the International Union of Biochemistry is used):

codY-F3: 5'- ctt tta gaa att acg tcc wgc -3'

codY-R3: 5'- cct ttc ata cca agt gaa cgt g -3'

Genomic DNA of *L. sanfranciscensis* DSM20451^T was used as template. PCR amplificates with the expected size were isolated from the gel using an E.Z.N.A Gel Extraction kit (Peqlab Biotechnologie GmbH) and sequenced by Sequiserve (Vaterstetten, Germany).

2.6 Cystathionine lyase activity in lactobacilli

2.6.1 Screening lactobacilli genomes for a cystathionine lyase gene

Degenerated primers targeting *cxl* were designed based on conserved sequences of *cbl* of *L. reuteri* (AJ293860), and *cgl* from both *Bacillus subtilis* (U93874) and *L. fermentum* (U97348).

The following primer pair was used (for mixed base sites, the code of the International Union of Biochemistry is used):

Ly-F3: 5'-gay gtn tay ggn ggn ac-3'

Ly-R2: 5'-ytc dat ncc ncc nar nsw ytg-3'

Genomic DNAs of all strains that are listed in table 2 were used as templates. PCR amplificates with the expected size were isolated from the gel using an E.Z.N.A Gel Extraction kit (Peqlab Biotechnologie GmbH) and sequenced by Sequiserve (Vaterstetten, Germany).

2.6.2 Cystathionine lyase expression by lactobacilli

The obtained cystathionine lyase sequences (see section 2.7.1) were used for primer improvement. The following primers were used:

Ly-F5: 5'-gaa acd cci acy aay cc-3' (i: inosine)

Ly-R4: 5'-gtg gcr swg tga ada c -3'

2.7 Glutamine deamidation by lactic acid bacteria

2.7.1 Glutamine and glutamate levels during sourdough fermentation

Wheat flour with an ash content of 0.55% was obtained at a local supermarket. To inoculate sourdoughs, 25 mL of an overnight culture were washed twice with tap water. The pellet was resuspended in 7.5 mL tap water and this cell suspension was mixed with 7.5 g flour and 0.08 g fungal protease PR59. This fungal protease preparation from *Aspergillus oryzae* is commercially available for use in baking improvers, contains a mix of proteinase and peptidase activities prepared and has a specific activity of 100 hemoglobin units g^{-1} (acid protease on hemoglobin substrate). The doughs were fermented at 30°C for 24h. Glutamine and glutamate levels in the fermented doughs were measured by HPLC as described in section 2.1.5.

2.7.2 Role of glutamate in bread flavor

Chemically acidified sourdoughs were prepared from 280 g wheat flour (type 550), 280 g tap water, 2.016 mL lactic acid and 0.504 mL acetic acid. The doughs were incubated overnight at 30ºC. Reference bread doughs (dough yield 160) were prepared using white wheat flour (750 g), tap water (405 g), fresh, compressed yeast (20 g), salt (10 g) and chemically acidified dough (225 g). The sample bread doughs contained additional glutamate (mono sodium salt, 3.0 g, 1.8 g or 0.8 g). The doughs were kneaded for 3 minutes, proofed for 40 minutes and baked at 220°C for 45 minutes.

Before sensory analysis, the bread was allowed to cool down for 1 hour. Triangular tests were performed with pieces of bread crumbs as described in section 2.2.2.

2.7.3 Glutamine deamidation activity of lactobacilli

For each strain, two separately overnight grown cultures were harvested and washed twice with potassium phosphate buffer A (50mM, pH 7.0). Subsequently, cell suspensions (OD_{590} = 10.0) were prepared and diluted 1:1 with in potassium phosphate buffer B (50mM, pH 7.0; 40 mM glutamine, 5 g L^{-1} glucose). For *L. sanfranciscensis* DSM20451^T, buffer A and B were replaced by buffer C (imidazole, 50mM, pH 5.50) and D (imidazole, 50 mM, pH 5.50, 40 mM glutamine, 5 g L^{-1} maltose), respectively. The samples were incubated at 37°C for 0, 0.5, 3 and 6 hours. Freezing the samples stopped the reaction. Substrate blanks and cell suspensions that were inactivated by heating at 90°C for 7 minutes were included. Deamidation of glutamine was monitored by measuring glutamic acid levels using a 'glutamic acid detection kit' (R-Biopharm, Darmstadt, Germany). The method that provided by the manufacturer was downscaled to a volume of 250 µL and the absorbance of the reaction mixture was measured at 492 nm in a microtiterplate spectrophotometer (TECAN spectrafluor, Grödig, Austria).

2.7.4 Glutaminyl residue deamidation by lactobacilli using gliadins as substrate

Gliadin proteins were extracted from white wheat flour (type 550) essentially according to Thiele et al.[53]. Five g wheat flour was sequentially extracted with 20 mL of the following solvents: (1) deionized sterile water, (2) 0.5 M NaCl in 150 mM NaH_2PO_4, pH 6.8, and (3) 50% (v / v) 2- propanol in water. Each extraction step performed at room temperature for 30 min, the suspension was thoroughly mixed every 10 minutes. Between solvent 2 and solvent 3, the pellet was washed with deionized water in order to remove salts. The extraction step with solvent 3 was performed twice, and the 2 extracts were pooled and freeze-dried. The gliadins were stored at 4°C.

The buffer fermentation was performed as described in section 2.7.2, the glutamine in the reaction buffer was replaced by 2 mg mL^{-1} gliadin and OD590 of the cell suspension was 5 instead of 10. Deamidation was monitored by measuring the release of ammonium.

2.7.5 Determination of the deamidation of glutaminyl residues in α2 (58-88) gliadin-peptide

The α2-(58-88)-gliadin-peptide was kindly provided by Dr. Herbert Wieser (Deutsche Forschungsanstalt für Lebensmittelchemie, Garching, Germany). Overnight grown cultures of *L. sanfranciscensis* DSM20451^T and *L. reuteri* TMW1.106 were harvested and washed and the cells were resuspended to a final OD of 150 in imidazole (50 mM, pH 5.5) and potassium phosphate buffer (50 mM, pH 7.0), respectively. One mg α2-(58-88)-gliadin-peptide was solved in 140 µL reaction buffer and 20 µL cell suspension was added. The samples were incubated at 37°C for 6 hours. The fermentation was stopped by spinning down the cells and freezing the supernatants.

In a separate experiment, the cells were lysed after harvesting, cell debris was removed and to 600 µL of the cell extract, 1 mg of α2-(58-88)-gliadin-peptide was added. The reaction was stopped by the addition of perchloric acid to a final concentration of 3.5%. The samples were clarified by centrifugation and stored at –20 °C.

The α2-(58-88)-gliadin-peptide was chemically deamidated by incubating a peptide solution (0.5 mg mL^{-1}) in 2 M HCl at 50°C for 6 h. Samples were taken after 0, 2, 4 and 6 h and were used for ammonium determination and were diluted 1:1 before RP-HPLC analysis.

The chemically deamidated samples and microbial treated samples were injected on an RP-HPLC system equipped with a RP C18 column (Jupiter, 250 x 4.60 mm, 10 µm particle size, Phenomenex, Torrence, U.S.A.) coupled to a UV detector set to 210 nm. The flow was 1 mL / min, eluents A and B consisted of 0.1% trifluoroacetic acid in water and acetonitrile, respectively. Samples were eluted with the following solvent gradient: 0 to 10 min: 1% B, 10 to 50 min: gradient from 1 to 60% B, 50 to 70 min: gradient from 60 to 100% B, followed by elution with 100% B as a column wash, and re-equilibration to 1% B.

2.7.6 Ammonium determination

Fermentation samples were clarified and to 50 µL of the clear supernatant, one mL of each reagent A (140 g trisodium citrate and 5 g NaOH in 1000 mL deionized water), reagent B (35 g phenol and 0.40 g sodium nitroferricyanide (III) in 1000 mL deionized water) and reagent C (1 g dichloroisocyanuric acid (sodium salt) and 10 g NaOH in 500 mL deionized water) were added. The reaction mixture was incubated in the dark at room temperature for 2 hours. The absorbance was measured at 630 nm against a blank. Ammonium chloride was used for the calibration curve.

2.7.7 Determination of the pH_{opt} and T_{opt} for glutaminyl-residue deamidation by *L. reuteri* TMW1.106 cell extracts

Four different buffers were used in order to determine the pH_{opt} for the deamidation of glutaminyl residues in gliadins by *L. reuteri* cell extract; pH 5.0 (trisodium citrate, 50 mM), pH 6.0 (potassium phosphate, 50 mM) pH 7.0 (potassium phosphate, 50 mM), pH 8.0 (potassium phosphate, 50 mM) Cells of an overnight grown *L. reuteri* TMW1.106 culture were harvested by centrifugation and resuspended in the required buffer corresponding to the experiment and lysed by ultrasonication for 4 times 60 sec on ice (Bandelin UW2070 ultrasonication processor). Cell debris was removed by centrifugation. pH_{opt} for glutamine and glutaminyl-residue deamidation were determined in a mixture of 300 µL cell extract and 300 µL 40 mM glutamine or gliadin (2 mg mL^{-1}) in the required buffer, respectively. The reaction was performed at 37°C.

The optimum temperature was determined by incubating the reaction mixture (pH 7.0) at 30°C, 37°C and 45°C. The deamidation of glutaminyl residues was monitored by measuring the ammonium release (section 2.7.4).

2.7.8 Fractionation of *L. sanfranciscensis* DSM20451^T

Two liter modified mMRS were inoculated with L. sanfranciscensis and incubated for 80 hours. Cells were harvested by centrifugation. Supernatant was stored at -20ºC and the pellet washed with sodium phosphate buffer (50 mM, pH 7.0). The washed pellet was shock-frozen at -20ºC, and after thawing, the cells were treated with lysozym (70 mg/mL cell suspension) at 37ºC for 1.5h. Degradation of the cell wall was monitored by microscopy. Subsequently, cells were lysed by ultrasonication followed by ultrathurax treatment. The cell wall fraction was collected by centrifugation (40 min, 10.000 g, 4ºC), and the cell membrane fraction was separated from the cytoplasmatic fraction by ultracentrifugation (1h 20min, 128.000 g, 4ºC). Both cell wall and cell membrane fractions were resuspended in sodium phosphate buffer (50 mM, pH 7.0). Protein contents in the different fractions were measured using Bio-Rad Protein assay (Bio-Rad, Munich, Germany). Ten mL Bio-Rad reagent was diluted with 40 mL water. The diluted reagent (250 µL) was transferred into a microtiterplate, the wells of which already contained 2.5 µL sample, the absorbance was measured after 5 minutes at 590 nm in a microtiterplate spectrophotometer (TECAN spectrafluor, Grödig, Austria). A calibration curve was prepared using bovine serum albumin as standard.

2.7.9 Screening lactobacilli genomes for a glutaminase-gene

Degenerated primers targeting *glnA* were designed based on conserved sequences of *glnA* of *L. plantarum* WCFS1 (AL935256), *L. rhamnosus* (AJ224996), *L. sakei* 23K (NC007576), *L. casei* ATCC334 (draft of the complete genome on-line available at http://img.jgi.doe.gov) and *L. delbrueckii* (D10020). The following primer pair was used (for mixed base sites, the code of the International Union of Biochemistry is used):

glnAv4: 5'-ggycarcadgaaatygaytt-3'

glnAr4: 5'-gcrttgtgharvgtwtwtgg-3'

Genomic DNAs of *L. sakei* TMW1.22, *L. sanfranciscensis* DSM20451^T, *L .frumenti* TMW1.103, *L. reuteri* TMW1.106, *L. plantarum* TMW1.468, *L. perolens* TMW1.501, *L. fermentum* TMW1.890 and *W. confusa* TWM1.928 were used as templates.

3 Results

3.1 Metabolic potential of sourdough starter cultures

Preferments were prepared with the 24 strains of lactic acid bacteria that are listed in table 2. The selection of strains comprised representative organisms of those lactobacilli that are known to dominate sourdough fermentations and species that are found in other cereal 'fermentations' like spoiled beer. Furthermore, one organism isolated from duck intestine and one isolated from fermented sausage were included. The preferments were characterized with respect to cell counts, pH and levels of organic acids. The results are shown in table 7. All strains grew to cell counts exceeding 10^7 cfu g^{-1}, acidified the dough to pH-values ranging from 3.4 to 4.0, and produced lactate to levels in the range of 80 to 120 mmol / kg dough, indicating that the selected strains are suitable as starter cultures in sourdough fermentations. As an exception, *W. confusa* strains produced 50 – 60 mmol lactate / kg only, corresponding to a dough pH of 4.04, *L. perolens* strains produced more than 120 mmol lactate / kg and *L. perolens* TMW 1.500 decreased the dough pH to 3.36. Acetate levels were less than 10 mmol / kg in doughs started with homofermentative strains and 10 – 20 mmol / kg in doughs started with heterofermentative strains. The fermentation quotients (molar ratio between lactic and acetic acid) were calculated and are shown in table 7 as well. All homofermentative strain, except for *L. sakei* and *L. amylolyticus*, produced only small amounts of acetate, resulting in fermentation quotients over 20. In sourdoughs fermented with heterofermentative strains the fermentation quotient after 96h ranged from 3.8 to 12.0.

The concentrations of aroma relevant amino acids (leucine, isoleucine, valine, phenylalanine, methionine, cysteine, cystine) were determined for 15 strains and were compared to their respective levels in chemically acidified dough. The results are presented in figure 6. Generally, the concentration of aroma relevant amino acids increased during fermentation, in agreement with previous observations [7]. *L. reuteri*, *L. pontis*, *L. amylolyticus* and *L. fermentum* produced ornithine.

The sensory analysis of preferments by an untrained panel of three evaluators revealed a number of strains that produced a dough odor different from the odor of the sourdough reference, *L. sanfranciscensis* TMW 1.52 (table 8). The differing odor notes perceived were described as "fatty", "fruity", "buttermilk", "hay" or "sweaty". For most of these strains, it was confirmed by triangular tests with a trained sensory panel that the dough odor significantly differed from the odor of sourdough fermented with *L. sanfranciscensis* TMW1.52 (personal communication Dr. Michael Czerny, Deutsche Forschungsanstalt für Lebensmittelchemie, Garching, Germany).

Table 7. Cell counts, pH, and metabolites in preferments started with lactic acid bacteria

Species	TMW#	Lactate [a)]	Acetate [a)]	Ethanol [a)]	FQ [b)]	Cell count [c)]	pH
L. fermentum	1.890	103.7	11.7	48.3	8.9	9.4	3.51
L. sakei	1.22	74.9	6.7	n.dt.[d)]	11.2	8.7	3.66
L. panis	1.648	100.4	9.8	82.0	10.2	8.9	3.47
L. brevis	1.465	94.0	17.8	85.8	5.3	8.9	3.61
L. amylolyticus	1.1078	98.8	7.6	65.8	13.0	9.5	3.39
L. mucosae	1.81	81.0	10.7	57.5	7.6	8.60	3.53
W. confusa	1.928	57.9	13.1	47.9	4.4	6.5	4.03
W. confusa	1.327	54.0	14.4	44.8	3.8	7.8	4.04
L. pontis	1.397	111.8	18.6	76.6	6.0	9.3	3.67
L. plantarum	1.460	122.3	3.9	30.3	>20	9.0	3.43
L. plantarum	1.468	120.5	4.4	n.dt.	>20	9.3	3.44
L. mindensis	1.1206	99.6	0.4	2.1	>20	8.9	3.55
L. reuteri	1.106	113.1	16.1	97.6	7.0	9.4	3.62
L. reuteri	1.976	105.6	14.3	68.8	7.4	8.6	3.59
L. frumenti	1.103	96.4	11.0	70.1	8.8	8.7	3.67
L. frumenti	1.666	111.7	14.8	97.1	7.5	8.7	3.62
L. sanfranciscensis	1.52	149.1	12.5	105.0	12.0	8.3	3.50
L. sanfranciscensis	1.53	65.7	17.5	42.2	3.8	7.9	3.98
L. sanfranciscensis	1.392	103.6	20.5	74.6	5.1	7.5	3.49
L. sanfranciscensis	1.949	126.0	12.9	94.9	9.8	8.5	3.56
L. sanfranciscensis	1.727	134.2	11.6	110.8	11.6	7.5	3.50
L. sanfranciscensis	1.1149	118.8	12.7	90.4	9.3	9.3	3.46
L. perolens	1.500	125.8	1.7	1.7	>20	9.0	3.36
L. perolens	1.501	160.2	3.9	3.9	>20	9.1	3.46

[a)]: all concentrations in mmol / kg dough, [b)]: FQ: fermentation quotient, calculated as [lactate] / [acetate]
[c)]: log cfu / g, [d)]: n.dt.: not detected

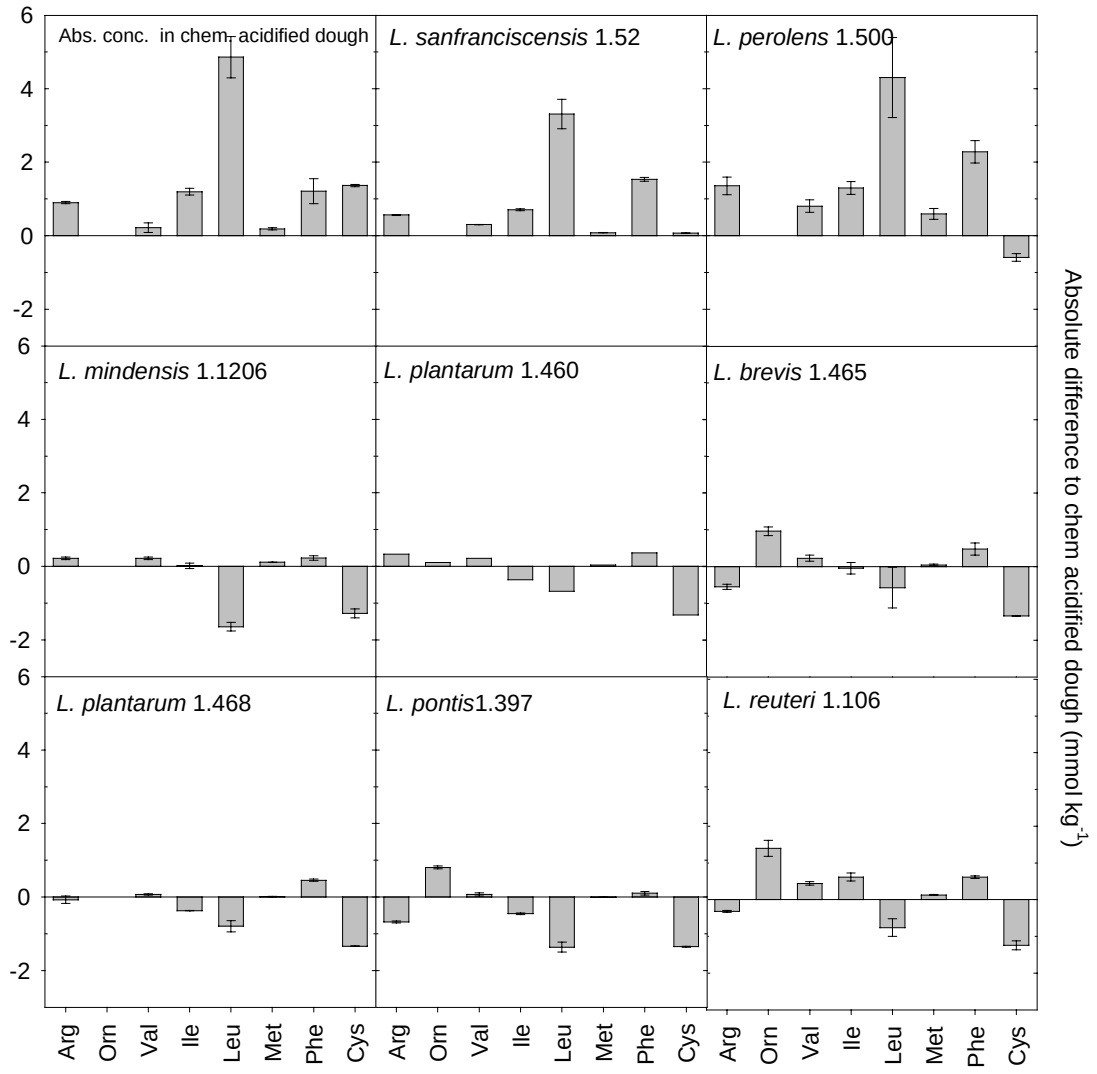

Figure 6a. Amino acids in sourdoughs. Concentration of flavor relevant amino acids in chemically acidified wheat dough (white wheat flour, type 550) after 96 h of fermentation, and differences of amino acid concentrations in sourdoughs to chemically acidified doughs. Shown are the results of two independent fermentations, each of which was analyzed in duplicate.

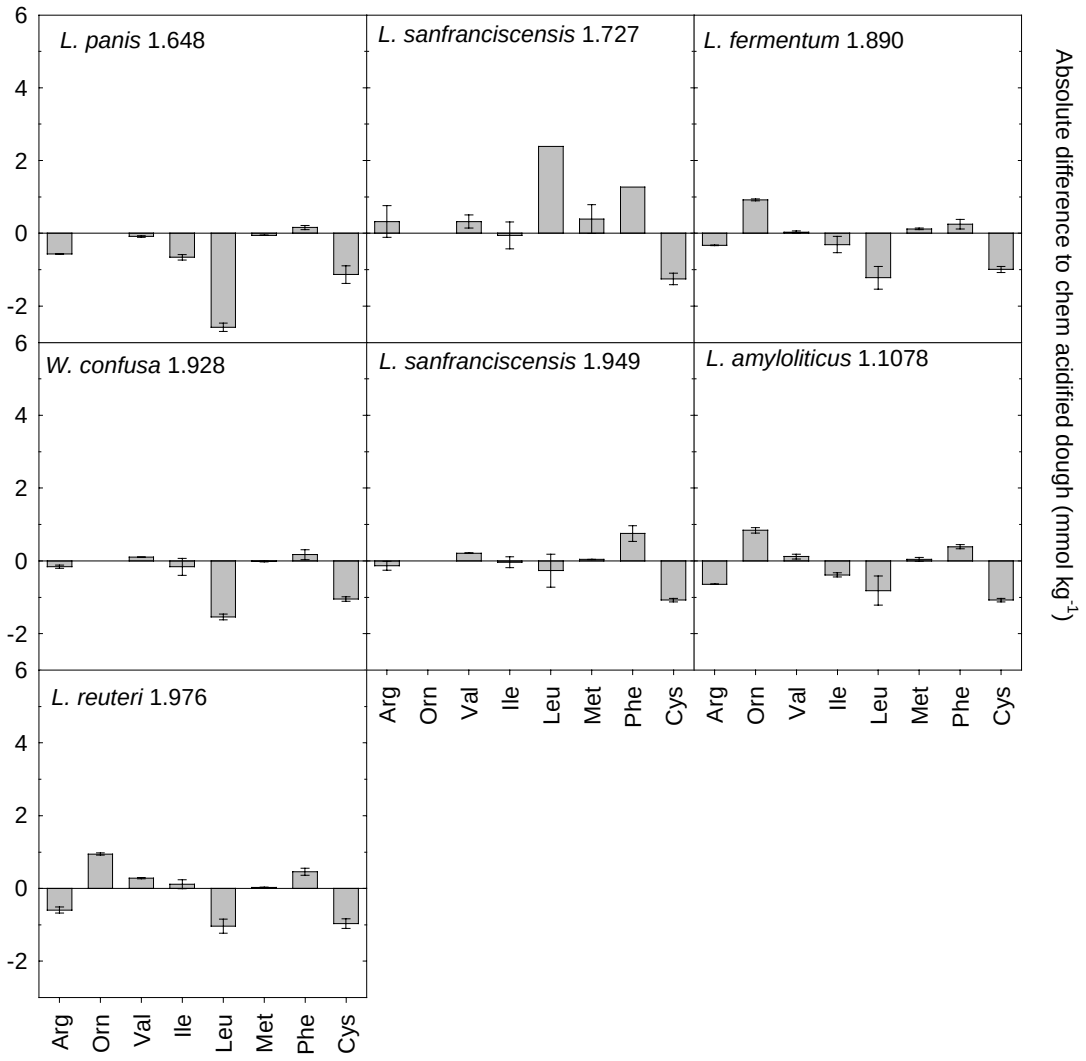

Figure 6b. Amino acids in sourdoughs, continued. Differences of flavor relevant amino acid concentrations in sourdoughs (white wheat flour, type 550, 96h of fermentation, 96h of fermentation) to amino acid concentrations in chemically acidified dough. Abolute concentrations of chemically acidified dough are given in Figure 6a. Shown are the results of two independent fermentations, each of which was analyzed in duplicate.

Table 8. Description of sourdough aroma. Descriptive analysis of the aroma of whole meal wheat flour-based sourdoughs fermented with lactic acid bacteria by an untrained panel (not perceived by all panelists)Tested were dough fermented with all strains listed in table 2, shown are descriptors of dough odors only when a difference was perceived to the reference dough fermented with *L. sanfranciscensis* TMW 1.52.

Organism	**TMW #**	**Characteristic odor**	
		1 day fermented dough	**4 days fermented doughs**
L. sanfranciscensis	1.727	(lemon)	hay
L. perolens	1.500 1.501	buttermilk	intense buttermilk
L. plantarum	1.468	fatty, fruity	fatty
L. pontis	1.397	(lemon)	
L. frumenti	1.103	buttermilk, banana	sweaty, fruity
L. reuteri	1.106		fatty
L. sakei	1.22	fatty	fatty, (fruity)
L. mucosae	1.81	card board	
L. mindensis	1.1206	buttermilk	buttermilk
L. panis	1.648		phenolic, (fatty)

In cheese and sausage fermentation, the addition of α-ketoglutarate causes an increase amino acid conversion [89;132]. This was investigated in doughs fermented with *L. sanfranciscensis* TMW1.52, *L. perolens* TMW1.501 and *L. sakei* TMW1.22. After 24 hours of fermentation, the added α-ketoglutarate was fully consumed, but no influence on amino acid metabolism was observed (data not shown). It was not possible to deduct which metabolic pathway was used for α-ketoglutarate utilization, because no distinct change in the formation of metabolites was observed. The addition of free amino acids to cheese [115] and sausage [114] improves the aroma of these products by enhanced production of amino acid metabolites. Thus, leucine was added to sourdough fermented with *L. sanfranciscensis* TMW1.52, *L. perolens* TMW1.501 and *L. sakei* TMW1.22, but no uptake was observed (data not shown).

3.2 Influence of citrate on the carbohydrate metabolism of *L. perolens*

Three out of three untrained evaluators described the odor of a 4 days old sourdough fermented with *L. perolens* or *L. mindensis* as 'buttermilk', it was assumed that this could be attributed to the formation of diacetyl. *L. perolens* has already been reported to produce diacetyl during beer spoilage [133]. In pastry and white wheat rolls, the consumer might consider a slight buttery odor pleasant. Diacetyl formation is closely related to citrate metabolism and α-acetolactate accumulation. Therefore, the influence of citrate on the metabolite α-acetolactate formation by *L. perolens* TMW1.501 was studied using both instrumental and sensory analyses. *L perolens* was chosen, since this strain is easier to grow under laboratory conditions then *L. mindensis* TMW1.1206.

3.2.1 Metabolite formation by *L. perolens* TMW 1.501 in laboratory medium and sourdough

L. perolens TMW1.501 was grown in mMRS medium in the presence of citrate and the concentrations of hexoses and metabolites were quantified and are shown in figure 7. Standard solutions of α-acetolactate cannot be purchased, because the compound is very unstable. The used standard was prepared from α-methyl-α-acetoxyethyl acetoacetate; since the reaction was not complete (see materials and methods section 2.1.4), it was impossible to quantify α-acetolactate, but the retention time was known and therefore, the production of α-acetolactate was only qualitatively measured (figure 7, 8). Diacetyl and acetoin were below their detection limits.

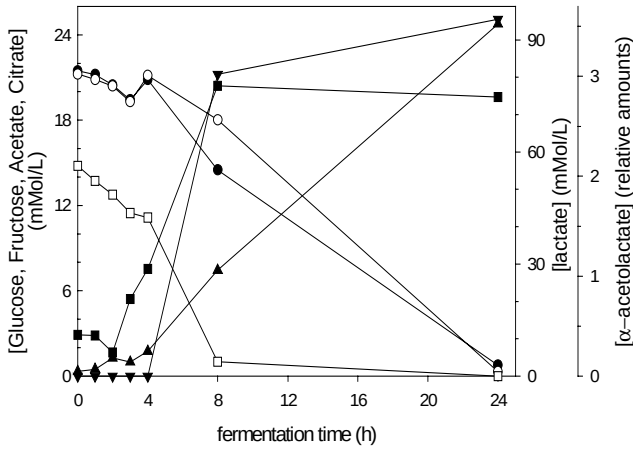

Figure 7. Metabolism of hexoses and citrate by *L. perolens* TMW 1.501 in mMRS medium. Shown are the concentrations of the substrates glucose (●), fructose (○), and citrate (□) as well as the concentrations of lactate (▲), acetate (■) and relative amounts of α-acetolactate (▼).

α-Acetolactate production was not observed in the absence of citrate, at aerobic conditions or during metabolism of pentoses in the presence or absence of citrate (data not shown). The addition of 15 mM citrate to mMRS medium caused an increase in acetate levels from 1.3 mM (data not shown) to 19.6 mM (figure 7). Furthermore, the addition of citrate did not influence the lag phase, but caused a significantly increased growth rate (data not shown). Low levels of α-acetolactate were produced during sourdough fermentation with *L. perolens* and α-acetolactate production was enhanced by the addition of citrate.

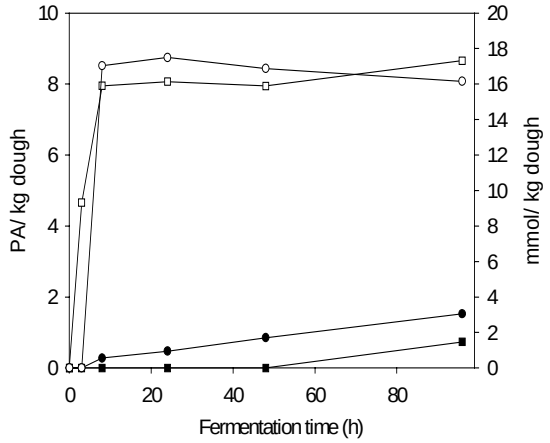

Figure 8. Formation of acetate and α-acetolactate in dough fermented with *L. perolens* TMW 1.501. Shown are the acetate concentrations (■) and relative amounts of α-acetolactate (PA, peak area, ●) in wheat doughs (filled symbols) and wheat doughs supplemented with 1.8 g citrate kg^{-1} dough (open symbols)

3.2.2 Sensory analysis

Triangular sensory tests were performed with sourdough fermented with *L. perolens* in the absence (reference doughs) and presence of citrate and with the corresponding bread crumbs. Untrained assessors were asked to identify the differing sample. According to Jellinek [120], a significant difference ($\alpha = 0.05$) was detected if 7 out of 10 panelists detected the differing sample; the result was highly ($\alpha = 0.01$) or if 8 or very highly significant ($\alpha = 0.001$), if 9 out of 10 assessors were able to detect the differing sample. The results of the triangular tests are shown in table 9. The addition of citrate to the preferment resulted in a very high significant difference in both preferment and bread crumb odor. Second, the panelists were asked to describe the difference in odor between the reference and sample sourdough and bread crumb, the results are presented in appendix 9.1. Whereas the odor of the preferment containing citrate was often described as more acidic or more like yogurt, buttermilk than the reference, the odor of the bread was described being more acidic only.

Table 9. Triangular tests of *L. perolens* TMW1.501-preferments and corresponding bread crumb. Reference preferments did not contain citrate, whereas 50 mM citrate was added to the sample preferments.

	Number of panelists that identified the differing sample [a)]	Significance [b)]
Preferment	10	$\alpha = 0.001$
Bread crumb	9	$\alpha = 0.001$

[a)]:10 panelists were questioned
[b)]: calculated according to [120]

3.3 Reduction of (E)-2-nonenal and (E,E)-2,4-decadienal during sourdough fermentation

Since (E,E)-2,4-decadienal and (E)-2-nonenal are key aroma compounds in the wheat bread crumb, the fate of these compounds during fermentation will greatly influence the flavor of the bread. The influence of the different microorganisms involved in the fermentation process on the levels of these odorants in dough was determined and the relevant metabolic pathways in these microorganisms were studied. One strain using the Embden-Meyerhof pathway for glucose metabolism (*L. sakei*), two heterofermentative strains (*L. sanfranciscensis* and *L. reuteri*) and baker's yeast (*S. cerevisiae*) were used as starter organisms.

3.3.1 Development of (E,E)-2,4-decadienal levels in fermented wheat doughs.

(E,E)-2,4-decadienal was quantified in wheat doughs fermented with either *L. sakei* TMW1.22, or *L. sanfranciscensis* TMW1.52, or *L. reuteri* TMW1.106 or *S. cerevisiae* TMW3.172. Considering the possibility of aldehyde generation during sourdough fermentation by flour lipoxygenase activity, chemically acidified dough was used as control. The results are shown in figure 9.

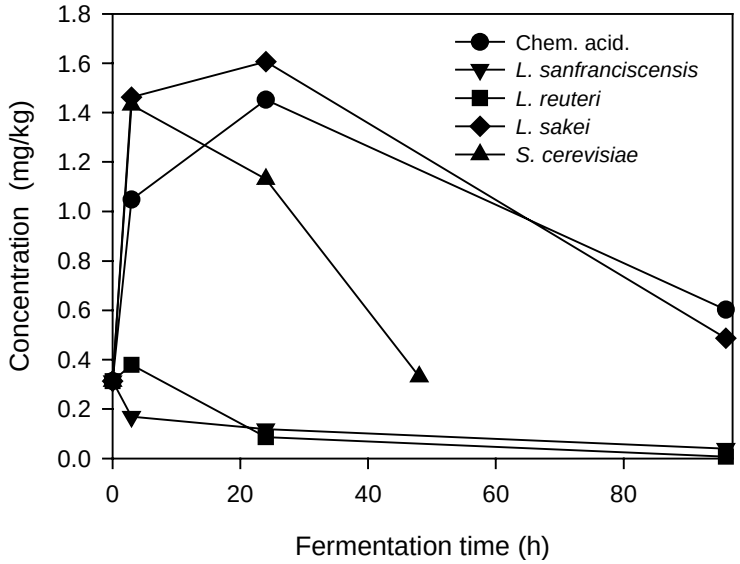

Figure 9. (E,E)2,4-decadienal levels in sourdoughs. Time course of the formation of (E,E)-2,4-decadienal in chemically acidified doughs and doughs fermented with *L. sanfranciscensis* TMW1.52 , *L. reuteri* TMW1.106 , *L. sakei* TMW1.22 and *S. cerevisiae* TMW3.172.

In the chemically acidified dough, the (E,E)-2,4-decadienal concentration increased during the first 24 hours. Continuation of fermentation up to 96 hours showed a decrease, indicating that (E,E)-2,4-decadienal is substrate in enzymatic or chemical reactions. Most likely, low levels of oxygen that are present in the dough cause ongoing lipoxygenase activity. In microbially fermented sourdoughs, oxygen will by efficiently removed by the microorganisms. In doughs fermented with *S. cerevisiae* or *L. sakei*, a slow decrease in (E,E)-2,4-decadienal levels was observed during the last 72 hours of the fermentation as well. In these two sourdoughs, the increase in (E,E)-2,4-decadienal levels during the first 8 hours was more pronounced as compared to the chemically acidified dough. Whereas the decrease of (E,E)-2,4-decadienal levels in dough fermented with *L. sakei* was similar to the chemically acidified dough, the decrease in the dough fermented with the yeast was faster. This indicates that in contrast to *L. sakei*, *S. cerevisiae* actively removed the (E,E)-2,4-decadienal. The aldehyde was removed quickly in the sourdoughs fermented with the heterofermentative strains *L. sanfranciscensis* and *L. reuteri*. Since no considerable increase in (E,E)-2,4-decadienal was observed during the first 8 hours, it was removed even faster than it originated from lipid oxidation.

3.3.2 Development of (E,E)-2,4-decadienal and (E)-2-nonenal levels in fermented wheat doughs.

To verify the different effects of yeast and homo- and heterofermentative lactobacilli on (E,E)-2,4-decadienal levels, and to determine whether a similar pattern is observed with (E)-2-nonenal, a second aroma-relevant aldehyde originating from lipid oxidation, the experiment was repeated in doughs spiked with both odorants. *L. sakei*, *L. sanfranciscensis* and *S. cerevisiae* were used as starter organisms. The aldehyde levels in fermented dough were monitored during 320 minutes, because the differences in concentration development were most pronounced during the first hours of fermentation. (E,E)-2,4-Decadienol and (E)-2-nonenol concentrations were quantified as well, in order to determine whether the aldehydes were reduced to the corresponding alcohols by the microorganisms. The results are shown in figure 10. In doughs fermented with *L. sakei,* only slight reductions of (E,E)-2,4-decadienal and (E)-2-nonenal levels were observed and the corresponding alcohols were not detected. Both *L. sanfranciscensis* and *S. cerevisiae* reduced the concentrations of the two aldehydes. In doughs fermented with *L. sanfranciscensis*, the decrease of (E,E)-2,4-decadienal and (E)-2-nonenal corresponded to an increase in (E,E)-2,4-decadienol and (E)-2-nonenol. In dough fermented with *S. cerevisiae* , the levels of the corresponding alcohol remained below the detection limit.

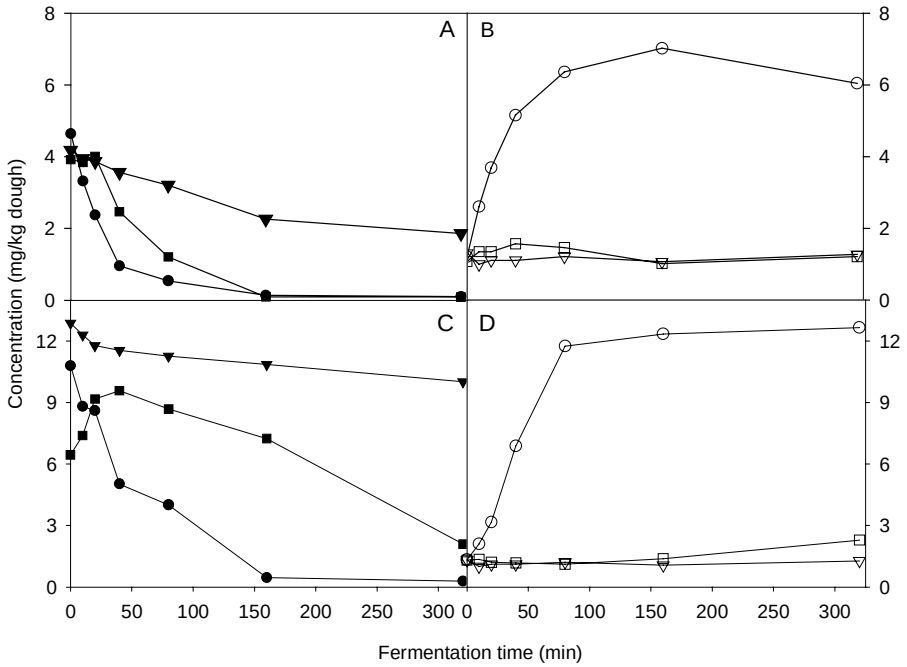

Figure 10. Aldehyde reduction during sourdough fermentation. (E)-2-Nonenal/-ol and (E,E)-2,4-decadienal/-ol concentrations in spiked doughs fermented with *L. sanfranciscensis* TMW1.52 (circle), *L. sakei* TMW1.22 (triangle), *S. cerevisiae* MW3.172 (square). **Panel A**: (E)-2-nonenal, **Panel B**: (E)-2-nonenol, **Panel C**: (E,E)-2,4-decadienal and **Panel D**: (E,E)-2,4-decadienol.

3.3.3 Reduction of (E)-2-nonenal by *S. cerevisiae* TMW3.172

Theoretically, (E)-2-nonenal can be the substrate for two different reductases: (i) the aldehyde moiety can be reduced to the alcohol by alcohol dehydrogenases and (ii) the α,β-unsaturated double bond can be hydrogenated resulting a saturated carbon-carbon bond. As displayed in figure 11, two possible conversion routes of (E)-2-nonenal to the saturated alcohol can be formulated based on these reactions: (i) reduction of the double bond to nonanal followed by reduction to nonanol (pathway 1) or (ii): reduction of the aldehyde function to (E)-2-nonenol followed by reduction to nonanol (pathway 2).

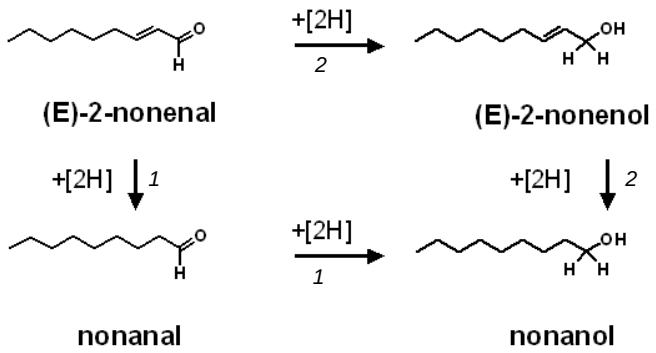

Figure 11. Conversion of (E)-2-nonenal to nonanol. Possible metabolic pathways for (E)-2-nonenal reduction by *S. cerevisiae*: Reduction of (E)-2-nonenal via nonanal (*1*) or (E)-2-nonenol (*2*) to nonanol.

It is unknown which reduction pathway is preferred by *S. cerevisiae* and *L. sanfranciscensis*, and which metabolites are formed. Therefore, experiments were carried out by fermenting wheat dough that was spiked with (E)-2-nonenal with the yeast and the concentration levels were observed by quantification of all four compounds. The results are shown in figure 12A. *S. cerevisiae* reduced the (E)-2-nonenal concentrations within 80 minutes. At that time point, (E)-2-nonenol levels were increased and equaled the (E)-2-nonenal levels, and a slight increase in nonanal concentration was observed as well. After 320 minutes, (E)-2-nonenal concentration was further decreased, (E)-2-nonenol levels were lower than after 80 minutes and nonanol levels were further increased.

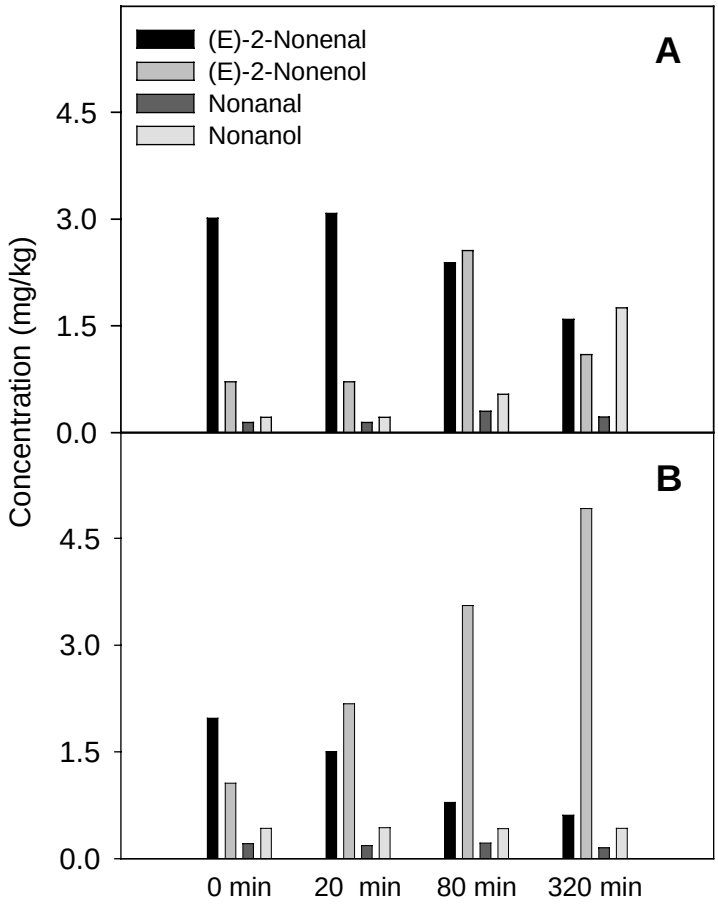

Figure 12. Concentrations of (E)-2-nonenal, nonanal, (E)-2-nonenol and nonanol in (E)-2-nonenal spiked doughs fermented with *S. cerevisiae* TMW3.172 (**Panel A**) and *L. sanfranciscensis* TMW1.52 (**Panel B**).

The concentrations of (E)-2-nonenal, (E)-2-nonenol, nonanal und nonanol (E)-2-nonenal-spiked doughs fermented with *L. sanfranciscensis* are shown in figure 12B. The final concentrations of (E)-2-nonenal and its metabolites as a sum were higher than the initial concentration of (E)-2-nonenal, indicating that in the dough, linoleic acid is oxidized to form (E)-2-nonenal. This makes it impossible to calculate carbon recoveries. In doughs fermented with *L. sanfranciscensis*, a decline in (E)-2-nonenal and a corresponding increase (E)-2-nonenol was observed. Compounds with reduced double bond did not emerge.

3.3.4 Influence of aldehyde reduction on glucose metabolism in *L. sanfranciscensis*

As a heterofermentative organism, *L. sanfranciscensis* energetically benefits from the utilization of external electron acceptors for the regeneration of reduced cofactor NADH because acetic acid can be formed from acetyl-phosphate with concomitant gain of additional metabolic energy. Acetic acid production in the presence and absence of an aldehyde was measured in a buffer system. Hexanal was used as electron acceptor because it has a better solubility compared to (E,E)-2,4-decadienal or (E)-2-nonenal. The results are shown in figure 13. Hexanal was reduced quantitatively to hexanol. In the presence of hexanal, higher acetic acid levels were measured, confirming a role of hexanal as electron acceptors.

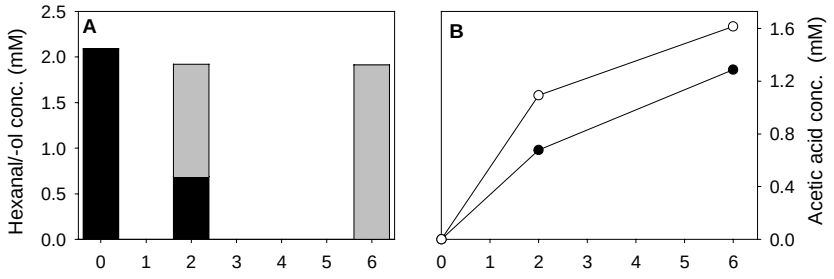

Figure 13. Hexanal reduction and acetic acid production by *L. sanfranciscensis* TMW1.52 during buffer fermentation. **Panel A:** Hexanal and hexanol concentration, **Panel B:** Acetic acid concentrations in a buffer with (open symbols, dashed line) and without (closed symbol, solid line) additional hexanal (2mM)

3.4 Influence of redox-reactions catalyzed by homo- and heterofermentative lactobacilli on gluten in wheat sourdoughs

The influence of lactobacilli on the oxidative cross-linking of the glutenins and thus on the polymerization of the GMP during sourdough fermentation was studied. First, the influence of enhanced enzyme activity and / or substrate availability on the formation of single amino acids was studied in chemically acidified doughs and microbially fermented sourdoughs. Second, thiol levels in wheat doughs were determined. Third, the amount of thiol-groups in gluten proteins was estimated by RP-HPLC separation of DACM-labeled propanol-soluble proteins extracted from wheat doughs. Finally, *L. sanfranciscensis* glutathione-reductase activity during growth in sourdough was investigated.

3.4.1 Effect of reducing agents and lactobacilli on proteolysis in wheat doughs

A reducing agent (dithiothreitol, DTT) and / or a protease were added to chemically acidified doughs. The concentrations of single amino acids were determined by HPLC and are depicted in figure 14A. In chemically acidified dough without additions, amino acids accumulated to 43 mmol / kg after 96 hours of fermentation. In the presence of DTT or protease, the levels of amino acids increased to 73 and 158 mmol / kg, respectively. Amino acid levels increased to 256 mmol / kg in doughs containing additional protease and DTT. Assuming an average molecular weight per amino acid of 110 g mol^{-1} and a flour protein content of 11%, it can be estimated that 52% of the wheat proteins were degraded to free amino acids during fermentation in the presence of protease and DTT. This clear synergy between DTT and the used protease indicates that both substrate solubility and enhanced proteolytic activity are needed for gluten degradation.

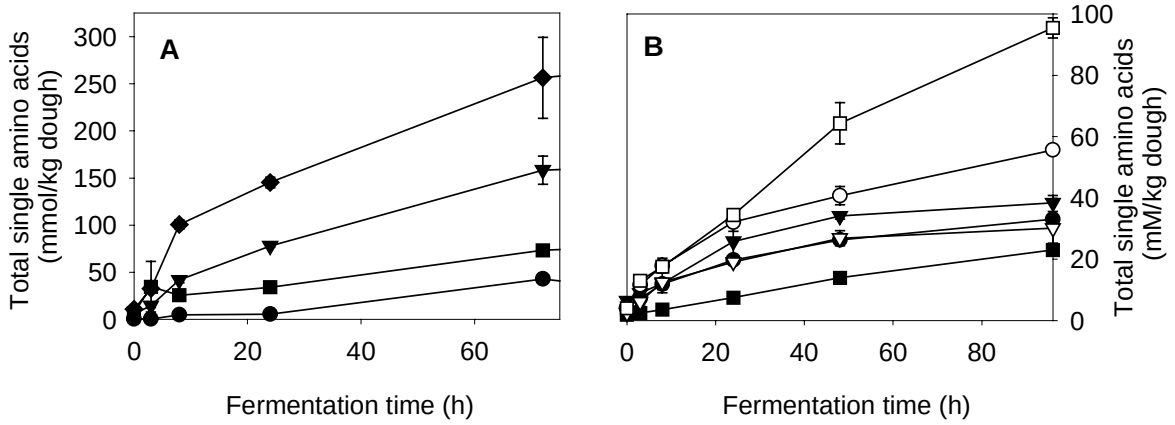

Figure 14: Development of total free amino acid concentrations during sourdough fermentation (flour type 550). **Panel A**: chemically acidified fermented doughs; untreated (●), with DTT (■), with industrial protease (▼) and with both DTT and industrial protease (◆). **Panel B:** microbially fermented doughs; filled symbols: microbially fermented dough; open symbols: with additional protease, *Lactobacillus sakei* TMW 1.22 (●), *Lactobacillus sanfranciscensis* TMW 1.52 (■), *Lactobacillus perolens* TMW 1.501 (▼). Data represent means ± standard deviation of duplicate independent experiments and the standard deviation was smaller than the symbol size when error bars are not shown.

On the one side, lactobacilli consume amino acids and they possibly contribute to proteolysis, either directly or indirectly. In sourdoughs, the amino acid levels did not exceed the levels in the chemically acidified dough (figure 14B). Thus, the proteolytic activity of lactobacilli did not exceed the consumption of low molecular weight nitrogen by the microorganisms. Upon protease addition to sourdoughs with *L. sakei,* the amino acid levels increased from 33 mmol / kg dough to 55 mmol / kg dough, in *L. perolens* sourdoughs, protease addition did not increase the levels of free amino acids. In contrast, in sourdoughs fermented with *L. sanfranciscensis* TMW1.52, an increase of the amino acid levels from 23 mmol / kg dough to 95 mmol / kg dough was observed upon protease addition. Since *L. sanfranciscensis* TMW1.52 does not display proteolytic activity, this difference between the strains can be explained by a higher peptidolytic activity of *L. sanfranciscensis* or by an increased substrate solubility based on the reduction of disulfide bonds by *L. sanfranciscensis* similar as the effect of DTT.

3.4.2 Influence of lactobacilli on the amount of free thiol groups in SDS-soluble protein fraction during sourdough fermentation

It was determined whether *L. sanfranciscensis, L. sakei* or *L. perolens* strains affect the concentration of reduced SH groups during sourdough fermentations. Free thiol groups in the SDS-soluble fractions of sourdoughs were quantified and their levels were compared to the concentrations in chemically acidified doughs and chemically acidified doughs with additional glutathione (GSH) (figure 15). Samples were taken from unfermented doughs after 5 hours of incubation, corresponding to exponentially growing cells in sourdough, and after 24 hours of incubation, corresponding to stationary cells. The thiol concentrations in dough are shown in figure 15.

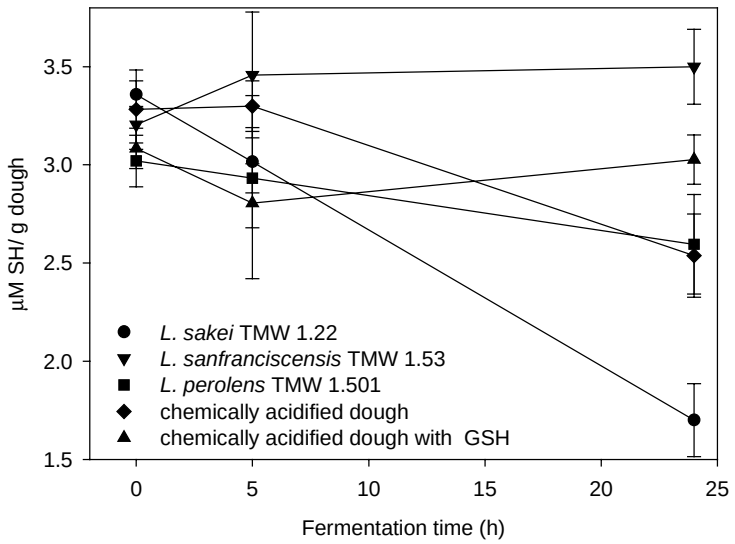

Figure 15. Free thiol groups in SDS-extracts from chemically acidified doughs and sourdoughs fermented with various strains of lactobacilli. Glutathione was added to chemically acidified dough to a concentration of 10 µmol g^{-1} dough. Data are means ± standard deviations of two independent fermentations analyzed in duplicate.

The differences in thiol levels between the unfermented doughs and after 5 hours of incubation were not significant. After 24 hours of incubation, in all doughs except for the *L. sanfranciscensis* TMW1.53 sourdough and the chemically acidified - GSH treated dough, the overall content of free thiol groups was decreased. The thiol content of sourdoughs fermented with *L. perolens* was similar as that of chemically acidified dough, whereas *L. sakei* sourdoughs contained less SH groups in the SDS-soluble protein fraction than chemically acidified doughs. *L. sanfranciscensis* TMW1.53 actively generated free thiol groups during sourdough fermentation, comparable to the thiol levels observed in the chemically acidified dough with added GSH. Virtually the same thiol levels were observed in sourdoughs fermented with *L. sanfranciscensis* TMW1.52 or TMW1.53 (data not shown).

3.4.3 Influence of *L. sanfranciscensis* on the amount of free thiol groups in propanol-soluble proteins during sourdough fermentation.

L. sanfranciscensis enhanced amino acid accumulation during sourdough fermentation and increased the amount of SH groups in the SDS-soluble fraction. Propanol-soluble proteins were isolated from sourdough fermented with *L. sanfranciscensis* for 24 hours and from chemically acidified control dough in order to determine whether redox-reactions catalyzed by *L. sanfranciscensis* TMW1.52 results in the reduction of disulfide bonds in gluten proteins. The SH groups in propanol-soluble proteins were labeled with DACM, a dye that specifically binds to SH groups, and separated with RP-HPLC coupled to a fluorescence detector. The protein content in the extracts was determined by analyzing the unlabeled fractions with RP-HPLC and UV-detection (210 nm). The chromatograms of the propanol-soluble protein fractions of the doughs after 0 and 24 hours are depicted in figure 16. In unfermented doughs, no appreciable differences were observed either in the protein concentrations or in the SH levels of propanol-extracts from fermented and chemically acidified doughs (figure 16A, B). In both doughs, the overall protein concentration was increased after 24h of fermentation. Extracts from *L. sanfranciscensis* TMW1.52 sourdoughs and chemically acidified doughs exhibited virtually identical protein concentrations as judged by the UV 210 nm trace (figure 16C). The peak at 26 mL disappeared and the peak areas between 18 and 23 mL were increased. In contrast, the fluorescence intensity of DACM-labeled propanol-soluble proteins did not increase in chemically acidified doughs (figure 16D). In *L. sanfranciscensis* TMW1.52 sourdoughs, the fluorescence intensity strongly increased after fermentation and especially proteins eluting between 20 and 25 mL exhibited strong fluorescence intensity. Qualitatively comparable results were obtained when commercial flour was used or when *L. pontis* was used (data not shown).

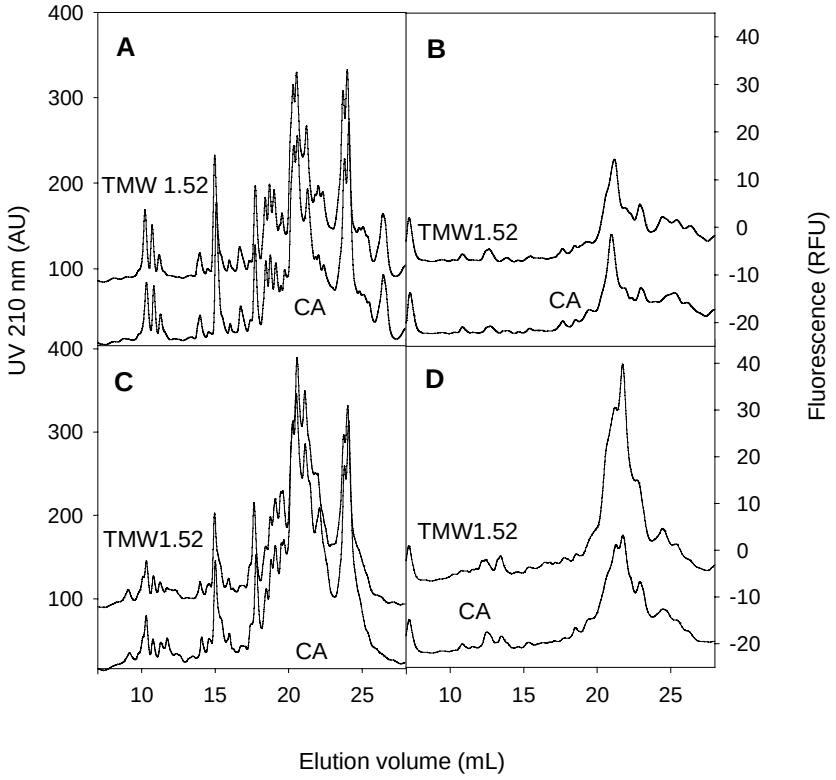

Figure 16: Separation of propanol-soluble proteins from 0h (A, B) and 24h fermented (C, D) sourdoughs and chemically acidified doughs by RP-HPLC. Left panels (A, C): UV-detection (210 nm) of unlabeled proteins. Right panels (B, D): Fluorescence detection of DACM-labeled proteins to quantify SH groups in the proteins. The data are representative for quadruplicate independent experiments.

3.4.4 Glutathione-dehydrogenase activity of *L. sanfranciscensis*

The activity of the glutathione reductase of *L. sanfranciscensis* may account for the increased SH levels in *L. sanfranciscensis* sourdoughs. Glutathione reductase catalyzes the oxidation of NADH to NAD concomitant with the reduction of the oxidized glutathione dimer (GSSG) to 2 GSH monomers. Cytoplasmic extracts of *L. sanfranciscensis* were shown to exhibit glutathione-reductase (GshR) activity [58], which was confirmed for the two strains used in this study (data not shown). It was determined in buffer fermentations with 10 mM GSSG whether the GshR of *L. sanfranciscensis* TMW1.53 catalyzes the reduction to extracellular GSH. After 2 and 6h of fermentation, 147 ± 7 and 73 ± 7 µM of GSH were formed from GSSG, respectively. Concomitant with GSSG reduction, the formation of acetate during 6h of fermentation increased from 140 ± 10 µM in the absence of GSSG to 255 ± 50 µM in the presence of 10 mM GSSG, linking GSSG-reduction to central carbon metabolism via the pentose-phosphate shunt. Only 1% of the GSSG present in the buffer were recovered as GSH, but GSSG levels were reduced by 10% during fermentation. This indicates that GSSG or GSH was additionally used as nitrogen source by *L. sanfranciscensis* in the buffer fermentations.

3.4.5 Glutathione reductase (*gshR*) expression during sourdough fermentation

The DNA-sequence of the gene coding for a glutathione dehydrogenase was retrieved in a partial genome sequencing of *L. sanfranciscensis* TMW1.53 [134]. *L. sanfranciscensis* TMW1.53 was grown in sourdough and samples were taken during the exponential phase (pH 4.8) and the stationary phase (pH 3.6). CopyDNA was synthesized and used as a template in PCR performed with *gshR* specific primers. The visualized PCR products are shown in figure 17. In both growth phases, *gshR* was expressed, indicating a contribution of this enzyme to increased SH-levels in dough.

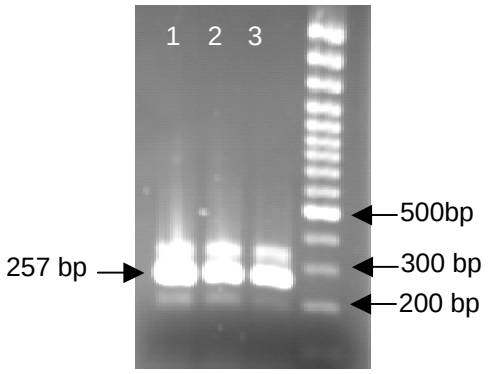

Figure 17: Expression of the glutathione dehydrogenase during growth of *L. sanfranciscensis* in sourdoughs. PCR with primers targeting *gshR* was carried out using the following templates: genomic DNA from *L. sanfranciscensis* DSM20451^T (lane 1), copyDNA from sourdough fermented with *L. sanfranciscensis* DSM20451^T after 5 hours (lane 2) and 24 hours (lane 3). The size of the PCR-products shown in lane 1, 2 and 3 match the size of the produce as predicted from the *gshR*-sequence (257 bp), and it was verified that the copyDNA-libraries were free of genomic DNA.

3.5 Proteolytic activity of *L. sanfranciscensis* DSM20451^T

In order to be able to intensify bread aroma by amino acid accumulation and microbial amino acid metabolism, extensive knowledge of the proteolytic system of lactobacilli is a needed. As discussed in the introduction, the proteolytic activity of lactobacilli during sourdough fermentation is controversial: on the one side, it was shown that microbial proteases play a minor role only during dough fermentation [7;51], on the other hand, it was shown that lactobacilli display enzyme activities that contribute to protein degradation during dough fermentation [70;71]. Therefore, *L. sanfranciscensis* was investigated with respect to proteolytic activity in three ways. The *L. sanfranciscensis* genome was screened for *prt* with degenerate primers targeting *prt* and proteolytic activity was analyzed both *in vitro* and during sourdough fermentation.

3.5.1 Screening for proteolytic activity of *L. sanfranciscensis* on genetic level

Sequences corresponding to Prt-proteinases carrying out protein hydrolysis in other lactic acid bacteria were not obtained on the genome preview. Therefore, chromosomal DNA of *L. sanfranciscensis* DSM20451^T was specifically screened for the presence of a *prt*-gene using 8 different primer combinations (table 5). All primer combinations amplified *prt*-fragments of appropriate sizes when DNA from *L. helveticus* TMW1.1255 harboring *prtH* or *L. lactis* NCDO 712 harboring *prtP* were used as template in the PCR reactions. Two of the PCR products were sequenced to ensure the suitability of the primer pairs used for the screening. PCR products of the appropriate sizes were not observed when

DNA from *L. sanfranciscensis* was used as template, and sequencing of unspecific PCR products did not reveal any similarity to *prt*-genes or other proteinases.

3.5.2 Screening for proteolytic activity of *L. sanfranciscensis* on biochemical level

To determine whether *L. sanfranciscensis* DSM20451^T exhibits proteolytic activity that enables the strain to use proteins as sole source of complex nitrogen, the strain was inoculated on N-limited mMRS (peptone and meat extract were omitted and 1 g L^{-1} yeast extract was added as sole source of complex nitrogen) or N-limited mMRS additionally containing 3 g L^{-1} casein, or 1.25 g L^{-1} peptone from casein. Growth was observed on N-limited mMRS in the presence of peptides, but not in the presence of casein only, or in the absence of an additional amino acid source (data not shown). This indicated that *L. sanfranciscensis* DSM20451^T does not exhibit proteolytic activity strong enough to enable the strain to grow on proteins as sole source of complex nitrogen.

3.5.3 Proteolytic activity of *L. sanfranciscensis* during growth in sourdough

Lactobacillus sanfranciscensis DSM20451^T was inoculated in wheat doughs in order to determine a possible influence of this strain on proteolytic events during sourdough fermentations. The strain grew to high cell counts in all doughs and the identity of the fermentation microflora with the inoculated strain was verified by observation of a uniform colony and cell morphology (data not shown), and specific PCR (see below). Pepstatin A was used to inhibit the proteolytic activity of endogenous aspartic proteinases, and fermented doughs were compared to chemically acidified doughs. In table 10, the levels of the metabolites lactate, acetate, and ethanol are shown. The metabolite levels indicated that pepstatin A had no appreciable inhibitory effect on growth and metabolism of *L. sanfranciscensis*. The acetate in the pepstatin A stock solution enhanced acetate levels in dough. Cell counts in chemically acidified doughs were below 10^5 cfu g^{-1}, excluding an impact of microbial metabolism on proteolytic events in these doughs.

Table 10. Metabolite concentrations in sourdoughs fermented for 24 hours with *L. sanfranciscensis* DSM20451^T. Presented results are mean values ± standard deviations of two separate dough fermentations.

[mmol kg^{-1}]	no additive	Pepstatin A 40 µM	Peptone 10 g kg^{-1}
Lactate	85.7±0. 5	74.5±1.6	88.4±1.5
Acetate	11.7±0.1	19. 9±1.1	11.0±0.1
Ethanol	68.3±0.6	43.5±2.4	54.7±0.8

Proteolysis was assessed by determination of amino nitrogen levels and the degradation of fluorescent casein. The amino nitrogen levels of fermented and chemically acidified doughs are shown in figure 18A. In chemically acidified doughs, amino nitrogen levels roughly doubled during incubation due to the activity of endogenous aspartic proteinases. The levels of amino nitrogen were higher in sourdoughs compared to chemically acidified doughs, but in both, the release of amino nitrogen during fermentation was virtually inhibited by addition of pepstatin A. The largest increase of free amino nitrogen was observed in sourdoughs with addition of peptides. In figure 18B, the changes in fluorescence during fermentation of doughs with fluorescent casein are depicted. In the chemically acidified dough, casein was degraded by endogenous flour proteinases, resulting in increased fluorescence in aqueous extracts of low-molecular weight peptides and amino acids. In agreement with previous results obtained with fluorescent gliadin and glutenin [52], dough fermentation with *L. sanfranciscensis* slightly increased protein degradation compared to the chemically acidified dough, but addition of pepstatin A inhibited protein degradation to a large extent both in chemically acidified and microbially fermented doughs. The release of peptides from fluorescent casein in chemically acidified doughs and sourdoughs with pepstatin A did not correspond to a similar increase of amino nitrogen (figure 18A and 18B). Large peptides have only one free amino nitrogen group detected by the ninhydrin staining, but may have several fluorescent groups. Therefore, residual proteolytic activity remaining in the presence of pepstatin A appears to produce rather large peptides. Taken together, proteolysis in the sourdoughs cannot be attributed to a serine proteinase of *L. sanfranciscensis*. Increased levels of amino nitrogen or amino acids in sourdoughs compared to chemically acidified doughs were observed only when cereal proteinases were active, i.e. in the absence of pepstatin A, and are thus attributable to the hydrolysis of peptides rather than proteins.

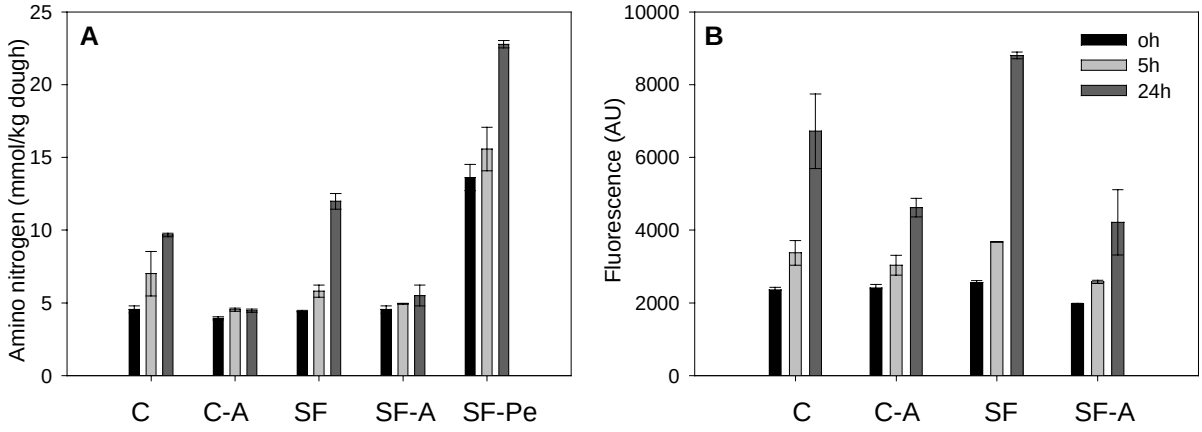

Figure 18. Proteolytic activity in sourdoughs fermented with *L. sanfranciscensis* DSM20451^T. The following doughs were analyzed: C: chemically acidified doughs, C-A: chemically acidified doughs with aspartic protease inhibitor, SF: *L. sanfranciscensis* sourdough, SF-A: *L. sanfrancsicensis* sourdough with aspartic protease inhibitor, SF-Pe, sourdough with addition of peptides. **Panel A:** Accumulation of low molecular weight amino nitrogen. Amino nitrogen was determined after 0, 5, and 24h of fermentation with a modified ninhydrin method. Presented are average values ± standard deviations of two separate fermented doughs. **Panel B:** Degradation of FITC-labeled casein. The fluorescence of perchloric acid-soluble degradation products was determined as arbitrary fluorescence units (AU) after 0, 5 and 24h of fermentation.

3.6 Formation of an intracellular amino acid pool by *L. sanfranciscensis*

Since the formation of an intracellular amino acid pool is the first step in amino acid metabolism, peptide transport and hydrolysis was studied in *L. sanfranciscensis* DSM20541^T. Genes involved in these two processes were identified and their expression levels during growth in sourdough were measured.

3.6.1 Identification of genes related to peptide transport and hydrolysis in *L. sanfranciscensis* DSM20451^T

Enzymes involved in peptide transport and hydrolysis can be considered of crucial importance, because *L. sanfranciscensis* ATCC27653 requires peptides for growth [72]. The genome of *L. sanfranciscensis* was partially sequenced [134] and, as shown in table 11, partial open reading frames (ORFs) were identified with high similarities to peptide-transport systems and peptidases of lactobacilli and *L. lactis*. Two peptide transporters, Opp and DtpT, and five peptidases, PepC, PepX, PepR, PepT, and PepN were identified. The gene coding for PepO contained several frame shifts, thus the gene product will not be functional (data not shown). In other lactobacilli, Opp is encoded by five

Table 11. Partial sequences from *L. sanfranciscensis* DSM20451^T coding for putative proteins involved in peptide transport or hydrolysis. Presented is the percentage of identical and similar amino acids as calculated by (http://dove.embl-heidelberg.de/Blast2/).

Hypothetical gene	Accession number DSM20451	Organism	Accession number reference gene	Identity (%)	Similarity (%)	Partial protein sequence DSM20451 (aa)	Size of full ORFs in databases (aa)
dtpT	AJ866920	*Lactobacillus plantarum*	AL935254	53	71	398	490
		Lactobacillus sakei	AB160861	55	72	399	454
oppF	AJ866923	*Enterococcus faecalis*	AE016956	70	86	238	314
		Lactobacillus delbrueckii	AY040221	68	88	238	318
oppD	AJ866924	*Lactobacillus johnsonii*	AE017204	67	82	239	336
		E. faecalis	AE016956	67	82	241	335
oppA	AJ866928	*L. johnsonii*	AE017204	52	71	120	586
		L. delbrueckii	AF496204	51	68	126	137
pepR	AJ866925	*L. sakei*	AF402317	69	83	217	300
		Lactobacillus rhamnosus	AJ003247	64	81	224	301
pepN	AJ866928	*L. johnsonii*	AE017204	74	89	271	362
		L. plantarum	AL935254	76	85	310	362
pepT	AJ866926	*L. plantarum*	AL935254	63	77	270	412
		Streptococcus mutans	AE014913	61	77	268	406
pepX	AJ866927	*Lactobacillus helveticus*	AB073633	45	59	379	803
		L. plantarum	AL935254	44	56	381	813
pepC	AJ866922	*L. plantarum*	AL935262	57	73	112	438
		L. helveticus	AY365130	50	73	116	437

genes (*oppD, oppF, oppB, oppC,* and *oppA*) with monocistronic organization [77]. In *L. sanfranciscensis,* the gene encoding PepN is located 150 base pairs downstream from *oppA* and a PCR approach using forward and reverse primers located on the *oppA* and *pepN* genes using copyDNA as template confirmed that these two genes are located on the same operon (data not shown).

3.6.2 Transcription of *pepR*, *pepC*, *pepX*, *pepT*, *opp-pepN* and *dtpT*, and regulation of *opp-pepN, dtpT* and *pepT* by *L. sanfranciscensis* during sourdough fermentation

The expression of genes involved in peptide uptake and hydrolysis was studied on transcriptional level, in order to investigate whether the peptide transport systems were relevant for peptide metabolism by *L. sanfranciscensis* during growth in sourdough. Transcripts of the genes *dtpT*, *oppF*, *pepC*, *pepR*, *and pepT* were detected in cDNA libraries from *L. sanfranciscensis* growing exponentially in sourdough, whereas transcripts of *pepX* were not detected. Doughs to which peptone from casein was added were analyzed in addition to doughs without additives in order to evaluate the role of the peptide supply on gene expression. Samples for total RNA isolation were taken during the exponential (pH 4.8) and the stationary growth phases (pH 3.6). The genes coding for peptide uptake systems, *oppF* and *dtpT*, and additionally one peptidase, *pepT*, were chosen for semi-quantitative transcriptional analysis. The regulation of gene expression in sourdoughs was investigated by quantification of the respective transcripts relative to *ldh* in the four cDNA libraries by using real-time PCR. The calculated relative expressions are shown in table 12. The expression of peptide transporters was highest in cells growing exponentially in dough without externally added peptides and the transcription of *dtpT* and of *opp-pepN* was reduced 31- and 71-fold, respectively, in the stationary phase. The *dtpT*- and *opp-pepN*-transcription by stationary cells in doughs with peptides equaled the transcription by stationary cells of *L. sanfranciscensis* in dough without peptides. However, the addition of peptides decreased the transcriptional levels of *dtpT* and *opp-pepN* in exponentially growing cells 16- and 18-fold, respectively.

The effects of the growth phase and the peptide supply on *pepT* transcription are much smaller than the effects on *dtpT* and *opp-pepN* transcription (table 12). In dough without additives, transcription was reduced 3.5-fold in stationary cells compared to exponentially growing cells. The addition of peptides decreased *pepT* expression in exponentially growing cells but in the stationary growth phase, the transcriptional levels of *pepT* were higher when peptides were present.

Table 12. Relative expression of genes related to peptide transport and hydrolysis by *L. sanfranciscensis* DSM20451 during growth in sourdough.

	growth phase	relative *oppF*-expression	relative *dtpT*-expression	relative *pepT*-expression
no additive	exponential	1	1	1
	stationary	0.014	0.032	0.284
peptone (10 g kg^{-1})	exponential	0.062	0.055	0.192
	stationary	0.018	0.033	1.167

3.7 Phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM20451^T and *Lactobacillus plantarum* TMW1.468

Bread spoilage is mainly due to the growth of filamentous fungi, and metabolites produced during sourdough fermentation by lactobacilli can inhibit fungal growth [97]. One of these metabolites is phenyllactic acid (PLA), which is a catabolite from phenylalanine (F) and is a flavor volatile as well (odor quality: honey). The influence of peptides and co-substrates on PLA formation from phenylalanine by *L. plantarum* TMW1.468 and *L. sanfranciscensis* DSM20451^T was studied.

3.7.1 PLA formation by *L. plantarum* and *L. sanfranciscensis* from different substrates

In all fermentations, the growth of the inoculated strains to high cell counts and the absence of a metabolically relevant number of contaminations were verified by observation of a uniform colony morphology on agar plates and by measuring lactate, acetate and ethanol levels in the doughs (data not shown). To determine whether amino acids or peptides are preferred substrate for amino acid metabolism, F was offered as a single amino acid or as part of a dipeptide. The concentrations of the peptides, F and PLA after 72h of fermentation are shown in figure 2. For both strains, a 2-4 fold increase in PLA formation can be seen when F is offered as part of a peptide compared to when F is offered as a single amino acid. Only when F is offered as part of FE, PLA formation is not increased in *L. plantarum* TMW1.468. In case of *L. sanfranciscensis* DSM20451, 0.0-0.38 mM of the initial 1.7 mM of the added dipeptides were recovered as dipeptides, thus, *L. sanfranciscensis* hydrolyzed 76% to 100% of the peptides. This caused an increase in phenylalanine concentration as compared to the fermentation without additional peptide: phenylalanine levels were 0.88 to 1.46 mM higher, indicating that a substantial part of the phenylalanine is not further converted. *Lactobacillus plantarum* hydrolyzed the dipeptide as well; after 72 h of fermentation, dipeptide concentrations ranged from 0.02 to 0.78 and phenylalanine levels increased with 0.44 to 1.38 mM. Phenylacetic acid, phenylethanol and phenylacetaldehyde concentrations were below detection limit after fermentation (data not shown). Kinetic analysis of growth rate and substrate and PLA, levels showed that peptide hydrolysis and PLA formation are ongoing when growth has ceased, indicating that amino acid conversion is not related to exponential growth but an ongoing process in stationary cells (data not shown). After 0h and 6h, PLA levels were below detection limit. After 24h, small amounts of PLA were formed but after 72h, PLA levels increased further, and the differences between strains and fermentation conditions were more pronounced and enabled statistical data analysis. For example, *L. sanfranciscensis* produced 0.048 mM PLA from FS and reached OD 0.75. After 72h, the OD remained essentially constant at 0.75 but PLA levels increased to 0.078 mM (data not shown). Due to low amounts of amino nitrogen available, only rather low optical densities were reached when lactobacilli are growing in N-limited medium.

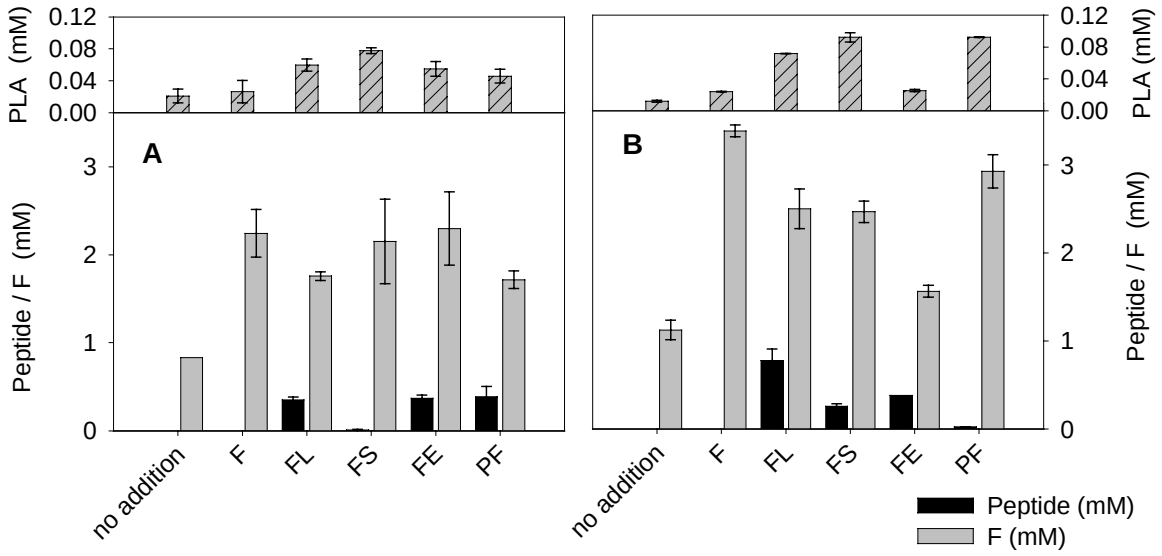

Figure 19. Peptide, F (lower panels) and PLA (upper panels) levels in nitrogen limited mMRS medium fermented for 72h with *L. sanfranciscensis* DSM20451^T (A) and *L. plantarum* TMW1.468 (B). Substrates were added to the medium to a concentration of 1.7 mM. Presented results are average values ± standard deviation of 2 independent fermentations.

3.7.2 Influence of α-ketoglutarate on the PLA formation by *L. plantarum* and *L. sanfranciscensis*

The accumulation of F indicates that peptide transport and hydrolysis are not a limiting factor, whereas the transamination is a bottleneck. In order to investigate whether the PLA formation can be enhanced by stimulation of the transamination, α-ketoglutarate and / or citric acid were added in excess, and substrate utilization and PLA formation (Figure 3) were monitored. As a control, α-ketoglutarate was added to medium without additional phenylalanine source but this addition did not enhance PLA formation significantly (data not shown). Independent of the supply with peptides and co-substrates, *L. sanfranciscensis* and *L. plantarum* grew to an optical density of 0.7-0.8 within 20h and the cell density remained essentially unchanged during further incubation. The addition of α-ketoglutarate strongly enhanced PLA formation in *L. plantarum*, the PLA yield increased from 5% to >30%. Corresponding to the increased PLA formation, decreased F levels were measured. The addition of citric acid did not enhance PLA formation further. Contrary, in order to stimulate PLA formation by *L. sanfranciscensis* the presence of both α-ketoglutarate and citric acid was a prerequisite. The achieved yield was not at all as high as the yield reached by *L. plantarum*, only 6% from the initially offered FS was recovered as PLA. This combined effect of α-ketoglutarate and citric acid was also observed when FL was used as substrate (data not shown). In addition to α-ketoglutarate, pyruvate may additionally act as amino acceptor to form alanine. However, none of the fermentations with *L. sanfranciscensis* and *L. plantarum* provided evidence for alanine accumulation through the use of pyruvate as amino acceptor.

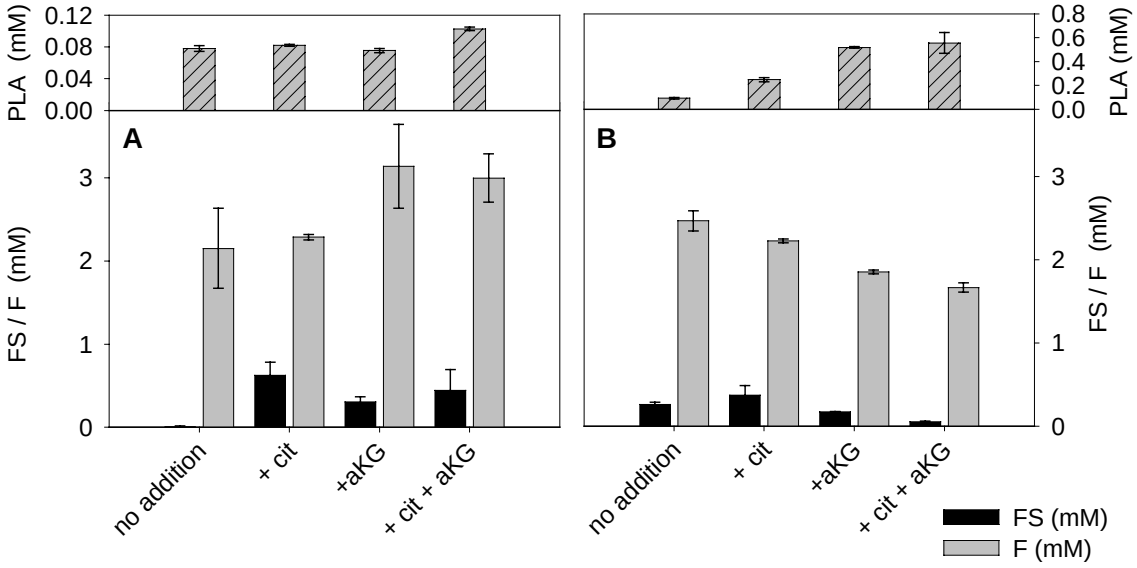

Figure 20. PLA formation by lactobacilli from different substrates. FS, F and PLA levels in nitrogen limited mMRS medium after 72h of fermentation with *L. sanfranciscensis* DSM20451^T (A) and *L. plantarum* TMW1.468 (B) are shown. FS (1.7 mM) was added at the start of the fermentation, as were citrate (cit, 5 mM) and α-ketoglutarate (aKG, 5 mM). Presented results are average values ± standard deviation of 2 independent fermentations.

Gdh activity enables generation of α-ketoglutarate from glutamate. The measurement of Gdh activities in cell-free extracts of both strains, revealed that *L. plantarum* had no active Gdh, whereas cell-free extracts from *L. sanfranciscensis* displayed both Gdh-NAD and Gdh-NADP activity, 1.8 nmol / (mg protein x min) and 19.5 nmol / (mg protein x min), respectively. In *L. sanfranciscensis*, α-ketoglutarate was exchanged by the single amino acid glutamate or by a glutamate-containing dipeptide. PLA levels in the medium were measured and are shown in figure 4. The combined effect of α-ketoglutarate and citric acid on amino acid metabolism was also observed when α-ketoglutarate was replaced by glutamate, either as a single amino acid or as part of a dipeptide, indicating that glutamate may substitute α-ketoglutarate in Gdh-positive strains. When glutamate was present, addition of α-ketoglutarate did not increase PLA formation further.

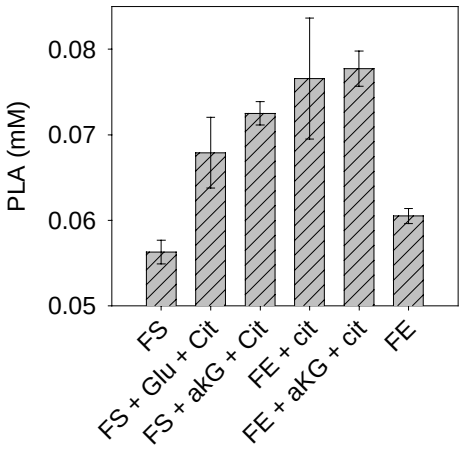

Figure 21. Influence of co-substrates on PLA formation by *L. sanfranciscensis* DSM20451^T. PLA levels were measured in nitrogen-limited mMRS medium containing either 1.7 mM FS or 1.7 mM FQ and co-substrates that was fermented for 72h with *L. sanfranciscensis* DSM20451^T. Used co-substrates were (glutamate: Glu; α-ketoglutarate: aKG; citrate: cit) were added to a concentration of 5 mM. Shown results are average values and standard deviation of 2 independent fermentations.

3.7.3 Stimulation of PLA formation in *L. sanfranciscensis*

Citric acid uptake and conversion may have several effects on the cell, i) its transport into the cell influences the membrane potential, an effect that is also described for the uptake and subsequent decarboxylation of malic acid [135], ii) the cometabolism of citric acid by *L. sanfranciscensis* results in the oxidation of 1 NADH to NAD, the same occurs when fructose is converted into mannitol [30]. The combined effect of α-ketoglutarate and various co-substrates on PLA formation was investigated in *L. sanfranciscensis*. The combination of citric acid and α-ketoglutarate did not influence the growth of *L. sanfranciscensis* (data not shown). Subsequently, citric acid was replaced by fructose or malic acid, and PLA formation was monitored in a buffer fermentation and in nitrogen limited MRS medium with 2 different substrates (figure 5). Malic acid did not enhance PLA formation, indicating that the membrane potential does not play a role in amino acid metabolism. With FL being the substrate, the addition of fructose resulted in significantly enhanced PLA formation (P<0.05), although not as strong as observed upon the addition of citric acid.

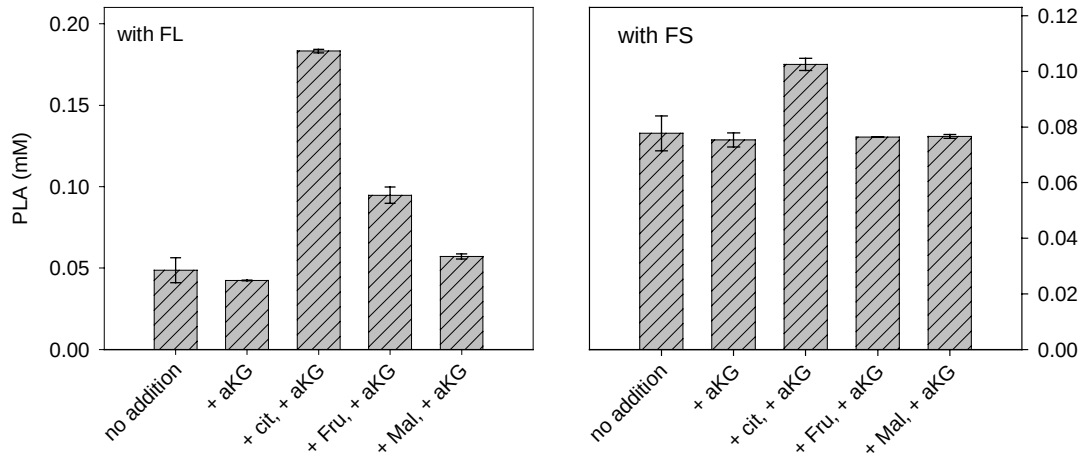

Figure 22. Influence of co-substrates on PLA formation by *L. sanfranciscensis* DSM20451^T PLA levels were measured in nitrogen-limited medium containing either 1.7 mM FL (left panel) or 1.7 mM FS (right panel) that was fermented for 72h with *L. sanfranciscensis* DSM20451^T. Additional substrates (fructose: Fru; α-ketoglutarate: aKG; citrate: cit; malate: Mal) were added to a concentration of 5 mM. Presented results are average values ± standard deviation of 2 independent fermentations.

3.7.4 Influence of branched-chain amino acids, citrate and α-ketoglutarate on the gene expression in *L. sanfranciscensis*

In *L. lactis*, the expression of genes coding for aminotransferases are inhibited by branched-chain amino acids via CodY [62;94], that binds to the DNA and prevents gene transcription. *L. plantarum* WCFS1 is devoid of *codY* [136] and degenerated primers targeting *codY* did not yield a PCR product when genomic DNA of *L. plantarum* TMW1.468 served as template (data not shown). In contrast, these primers yielded a PCR product when genomic DNA of *L. sanfranciscensis* was used. The PCR product was sequenced (gene sequence is shown in appendix 9.2) and translated into its amino acid sequence. The alignment of the amino acid sequence with other CodYs is shown in figure 23. Using SWISS PDB viewer, a DNA-binding helix-turn-helix motif was found. It can be concluded that *L.*

sanfranciscensis is *codY*- positive, and that the gene product is most likely able to bind to DNA. The amino acid sequence of $CodY_{L.\ sanfranciscensis}$ differs in 1 amino acid from the $CodY_{L.\ lactis}$ sequence. In *L. sanfranciscensis*, Opp and several peptidases are down regulated upon peptide addition and the used strain was *codY*-positive, which gave rise to the hypothesis that the expression of aminotransferases is influenced by the concentration of branched-chain amino acids in *L. sanfranciscensis*. Two aminotransferases were found on the partial genome sequence of *L. sanfranciscensis* DSM20541^T (raw data see [134]), the results of the WU-BLAST2-searches with post-processing at EMBL are shown in table 13. The influence of (i) the addition of FL (ii) a combination of citrate and α-ketoglutarate (iii) all three substances on the expression of *opp*, *araT1* and *araT2* in nitrogen limited mMRS medium was studied on transcriptional level. The *araT1-*, *araT2*- and *oppF* transcripts were quantified relative to *xpk* in the cDNA libraries by using real-time PCR and the obtained relative expression are shown in table 14.

```
sanfranciscensis      VATLLEKTRKITAILQDG-VTDLQQELPYNSMTERLANVIDCNACVINTKGELLGYSLPY 59
delbrueckii           ---LLEKTRKITAILQDG-VTDLQQELPYNSMTERLANVIDCNACVINTKGELLGYSLPY 56
lactis                MATLLEKTRKITAILQDG-VTDLQQELPYNSMTERLANVIDCNACVINTKGELLGYSLPY 59
enterococcus          MATLLEKTRQVNELLQKNNLFDVQAELPYNKMAMILGDILESNAYIISSSGDLLGYTEKL 60
                       ::******::. :**.. : *:* *****.*:  *.::::.** :*.:.*:****:

sanfranciscensis      NTNNDRVDQFFYDRKLPDEYVRAAVRIYDTMANVPVDRPLAIFPEESLSDFPKGVTTLAP 119
delbrueckii           NTNNDRVDQFFYDRKLPDEYVRAAVRIYDTMANVPVDRPLAIFPEESLSDFPKGVTTLAP 116
lactis                NTNNDRVDQFFYDRKLPDEYVRAAVRIYDTMANVPVDRPLAIFPEESLGDFPKGVTTLAP 119
enterococcus          DVNNARIKNMFKEKKFPQGYTEAVDMLKVTEANIPIDSDLTAFPFESRELYPFGLTTIVP 120
                      :.** *:.::* ::*:*: *..*.  :  * **:*:*  *: ** **   :* *:**:.*

sanfranciscensis      IYGSGMRLGTFIMWREDGEFTDDDLVLVELATTVIGVQLSNLKLEQMEENIRKDTMATMA 179
delbrueckii           IYGSGMRLGTFIMWREDGEFTDDDLVLVELATTVIGVQLSNLKLEQMEENIRKDTMATMA 176
lactis                IYGSGMRLGTFIMWREDGEFTDDDLVLVELATTVIGVQLSNLKLEQMEENIRKDTMATMA 179
enterococcus          LYGAGKRLGTIILARVEKSFNEDDLVLAEYSATVVGMQILYHQSRTIEAEVRSATAVQMA 180
                      :**:* ****:*: * : .*.:*****.* ::**:*:*:   : . :* ::*. * . **

sanfranciscensis      VNTLSYSEMKAVKAIIEELDGEEGHVIASVIADKIGITRSVIVNALRKLESAGVIESRSL 239
delbrueckii           VNTLSYSEMKAVKAIIEELDGEEGHVIASVIADKIGITRSVIVNALRKLESAGVIESRSL 236
lactis                VNTLSYSEMKAVKAIIEELDGEEGHVIASVIADKIGITRSVIVNALRKLESAGVIESRSL 239
enterococcus          INTLSYSELKAVHAIFEALDGEEGRLTASSIADEIGITRSVIVNALRKLESAGIIESRSL 240
                      :*******:***:**:* ******:: ** ***:******************:******

sanfranciscensis      GMKGTYLKVLNTGLFDKLAGRNF 262
delbrueckii           GMKGTYLKVLNTGLFDKLAGRNF 259
lactis                GMKGTYLKVLNTGLFDKLAGRNF 262
enterococcus          GMKGTYLKVLNQQFIKELEK--- 260
                      **********  ::.:*   .
```

„ ***** " : the residues in that column are identical in all sequences in the alignment.
„ **:** " : conserved substitutions have been observed
„ **.** " : semi-conserved substitutions

Figure 23. CodY-sequence allignment. Depicted is the multiple sequence alignment of the amino acid sequences derived from *codY* of *Enterococcus faecalis* V583 (NC004668, assigned 'enterococcus'), *Lactococcus lactis* subsp. *lactis* IL1403 (AE006253, assigned 'lactis'), *Lactobacillus delbrueckii* subsp. *lactis* (AX118803, assigned 'delbrueckii') and the gene product obtained using *codY* specific primers and *L. sanfranciscensis* TMW1.53 genomic DNA as template (assigned 'sanfranciscensis'). Underlined amino acids are predicted to be part of the DNA-binding helix-turn-helix motif. The amino acid printed on black background is the amino acid where the $CodY_{L.\ sanfranciscensis}$ differs from $CodY_{L.\ lactis}$.

Table 13. Partial sequences from *L. sanfranciscensis* DSM20451^T coding for putative aromatic aminotransferases. Presented is the percentage of identical and similar amino acids as calculated by (http://dove.embl-heidelberg.de/Blast2/).

Hypothetical gene	Organism	Accession number reference gene	Identity (%)	Similarity (%)	Partial protein sequence DSM20451^T (aa)	Size of full ORFs in databases (aa)
AraT1	*Lactococcus lactis* subsp. *cremoris*	AF146529	57	77	147	391
	Lactobacillus plantarum	AL935255	60	77	138	395
AraT2	*Carnobacterium piscicola*	AY029372	56	75	137	393
	Lactococcus lactis subsp. *cremoris*	AF146529	52	76	139	391

Compared to the changes in expression levels upon the addition of peptides to sourdough, the changes observed in table 14 are very small; no significant up- or down-regulation is observed upon the addition of FL or a combination of citrate and α-ketoglutarate. Thus, the stimulation of transamination by citrate and α-ketoglutarate is more likely to be caused on biochemical level than on genetic level.

Table 14. Expression of genes coding for the oligopeptide transporter Opp and 2 aromatic aminotransferases (AraT1 and AraT2) by *L. sanfranciscensis* DSM20451^T during exponential growth in N-limited mMRS medium.

Additive	relative *oppF*-expression	relative *araT1*-expression	relative *araT2*-expression
No additive	1	1	1
FL [a)]	1.625	1.314	1.493
Cit, αKG [b)]	0.857	0.362	0.628
FL, cit, α-KG	0.759	0.640	0.826

[a)]: FL: phenylalanine-leucine, final concentration 1.7 mM
[b)]: Cit: citrate, αKG: a-ketoglutarate, initial concentration 5 mM

3.7.5 Influence of branched-chain amino acids on the PLA formation by *L. sanfranciscensis*

The influence of increased intracellular branched-chain amino acids on PLA formation by, *L. sanfranciscensis*, which harbors *codY*, was investigated. Various combinations of dipeptides were added to the medium. PLA levels after 72h of fermentation are shown in figure 6. Neither the addition of LV nor the addition of LP to FS containing medium decreased the PLA formation. When 50% of FS was replaced by PF, FL or FE, no significant change (P>0.05) in PLA levels were measured.

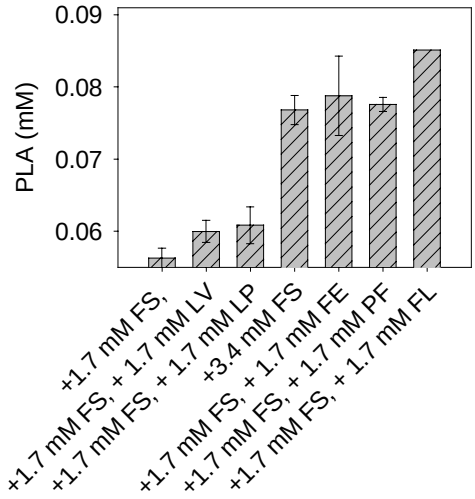

Figure 24. PLA levels in nitrogen-limited mMRS medium containing additional peptides fermented for 72h with *L. sanfranciscensis* DSM20451^T. Presented results are average values ± standard deviation of 2 independent fermentations.

3.7.6 PLA formation by *L. sanfranciscensis* and *L. plantarum* during sourdough fermentation

Sourdough fermentations were performed in order to examine whether the results obtained in medium can be transferred to PLA formation in sourdough. *Lactobacillus plantarum* was grown in a standard dough, and in doughs containing additional F, FS and both α-ketoglutarate and FS. *Lactobacillus sanfranciscensis* was grown in doughs with and without the addition of F, FS, and FS combined with citric acid, α-ketoglutarate and fructose. The PLA levels reached after 48h are shown in figure 7. Proper growth of the strains and absence of a metabolically relevant number of contaminations were verified by observation of a uniform colony morphology on agar plates (data not shown) and by measuring lactate, acetate and ethanol levels in the doughs (data not shown). PLA is formed during dough fermentation and its production was clearly increased when F or FS were added (P<0.1). In contrast to in N-limited medium, there was no significant difference detectable between the use of a dipeptide or a single amino acid. PLA production by *L. plantarum* in dough was enhanced when α-ketoglutarate was added. When *L. sanfranciscensis* was used as starter culture, addition of α-ketoglutarate and citric acid was needed in order to increase PLA production from FS significantly (P<0.05). This effect was not observed when fructose was added to the dough. A combined effect of citric acid and α-ketoglutarate but not of fructose and α-ketoglutarate was also observed when FL was added to the sourdough (data not shown).

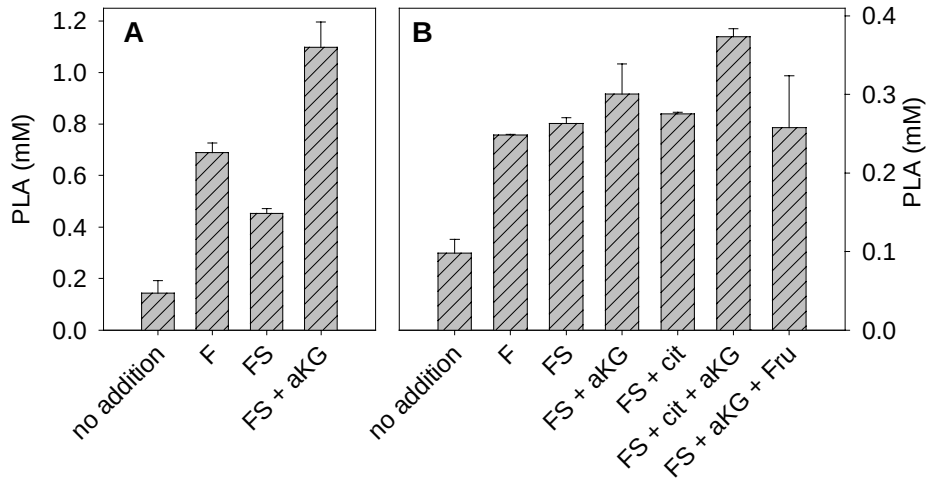

Figure 25. PLA levels in sourdough fermented for 48h with *L. plantarum* TMW1.468 (A) and *L. sanfranciscensis* DSM20451^T (B). Initial concentration of F, FS, α-ketoglutarate (aKG), citrate (cit) and fructose (fru) was 5 mmol kg^{-1} dough each. Presented results are average values ± standard deviation of 2 independent fermentations.

3.7.7 Antimicrobial activity of PLA

In order to investigate which concentrations of PLA inhibit fungal germination and bacterial growth, several fungal spore suspensions and one bacterial culture were used to inoculate media containing different concentrations of PLA in the presence of different concentrations of lactic and acetic acids (molar ratio lactate / acetate was 3.2 to 1). Three bread spoiling mold species were used, *P. roqueforti*, *P. citrinum* and *A. niger*. Additionally, one rope forming *Bacillus subtilis* strain was included. The tolerance of *L. sanfranciscensis* against PLA was investigated as well. The results are shown in figure 26. Inhibition of mold germination only occurred at high PLA and / or high lactic and acetic acid concentrations. At 37.5 mM PLA, none of the molds germinated, independent from the concentrations of lactic and acetic acids. In lower concentrations, the antifungal activity of PLA was dependent on the levels of lactic and acetic acids. *L. sanfranciscensis* was more sensitive against PLA than the tested molds: approximately 2 mM PLA prevented growth. Compared to the molds and *L. sanfranciscensis*, *B. subtilis* was more sensitive to both PLA and lactic and acetic acids.

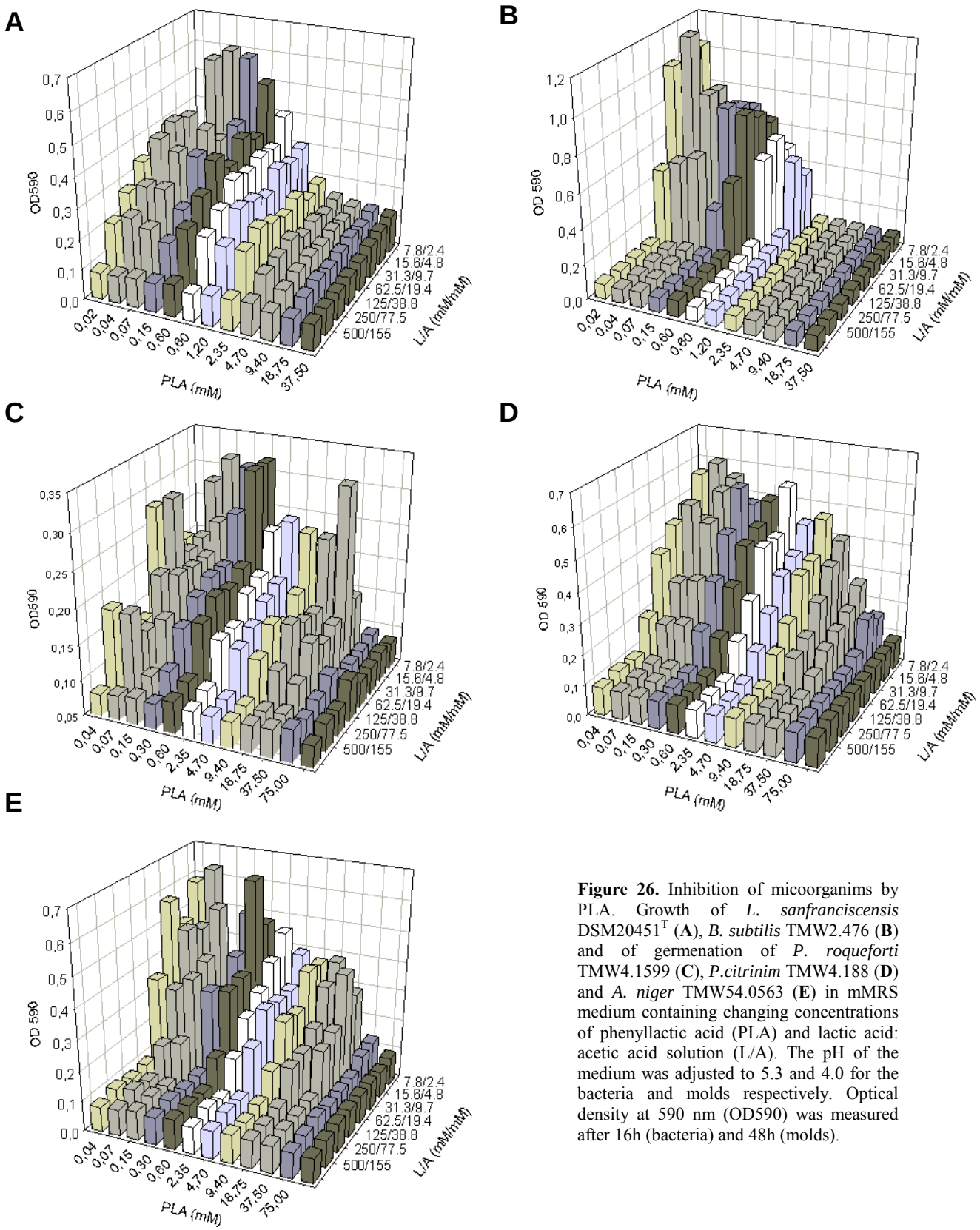

Figure 26. Inhibition of micoorganims by PLA. Growth of *L. sanfranciscensis* DSM20451^T (**A**), *B. subtilis* TMW2.476 (**B**) and of germenation of *P. roqueforti* TMW4.1599 (**C**), *P.citrinim* TMW4.188 (**D**) and *A. niger* TMW54.0563 (**E**) in mMRS medium containing changing concentrations of phenyllactic acid (PLA) and lactic acid: acetic acid solution (L/A). The pH of the medium was adjusted to 5.3 and 4.0 for the bacteria and molds respectively. Optical density at 590 nm (OD590) was measured after 16h (bacteria) and 48h (molds).

3.8 Metabolism of sulfur-containing amino acids by lactobacilli

3.8.1 Screening for cystathionine lyase activity in lactobacilli on a genetic level

The genomes of the selected lactobacilli were screened for cystathionine lyase (*cxl*), since cystathionine lyase is expected to play a role in flavor formation by lactic acid bacteria. All the strains listed in table 2 were screened for *cxl* on genomic level using degenerated primers. PCR products were obtained if genomic DNA of *L. fermentum* TMW 1.890, *L. pontis* TMW1.397, *L. panis* TMW1.648, *L. reuteri* TMW1.106, *L. reuteri* TMW1.976 and *L. mindensis* TMW1.1206 was used as template. The amplificates were sequenced; the alignment of the sequences with a known *cbl* gene from *L. reuteri* (AJ293860) and *cgl* gene from *B. subtilis* (U93874) are shown in figure 27. (In appendix 9.3, the gene sequences are shown). Distinguishing between cystathionine-β-lyase and cystathionine-γ-lyase based on amino acid sequences is not possible. The cystathionine lyase from *L. lactis* subsp. *cremoris* is even able to carry out both the α,β-elimination and the α,γ-elimination on cystathionine [104;137]. The crystal structure of cystathionine-β-lyase of *Escherichia coli* revealed which amino acids are essential for catalytic activity and cofactor binding [138]. It was verified that the essential amino acids were conserved in the partial sequences that were found in the course of this work (figure 27). The expression of *cxl* by cells growing in mMRS medium was assessed by mRNA isolation followed by reversed transcription and PCR using *cxl* specific primer. It was verified that no chromosomal DNA was co-isolated with the mRNA (data not shown). The results are shown in figure 28. It can clearly be seen that the gene is expressed by all tested strains.

```
1.890           ------------------------------------VHGGTFRLINKVLKRFGLEFTVVD 24
1.1206          ------------------------------------VHGGTFRLVNKVLKRFGLEFSVVD 24
1.397           ------------------------------------VYGGTFRLINQVLKRFGMEFMVVD 24
1.106           -----------------------------------DVYGGTFRLINKVLKRFGLEFTVVD 25
1.648           ------------------------------------VHGGTFRLINKVLKRFGLEFTVVD 24
1.976           ------------------------------------------------------------
Lb. reuteri     ELEHGTVGFAFASGSAAIHATFSLFSQGDHFVVGSDVYGGTFRLINKVLKRFGFEFTVVD 120
B. subtilis     ELESGEAGYAFSSGMAAITAVMMLFNSGDHVVLTDDVYGGTYRVMTKVLNRLGIESTFVD 120

1.890           TQDLAAVEAAVQDNTVAIYFETPTNPLLEVSDIEAIAKIAKKHGLKTVIDNTFATPYNQR 84
1.1206          TRDLQTVEAAIQPNTKAIYFETPTNPLLKITDIAAVAKIAKAHDLKTIVDNTFATPYNQN 84
1.397           TRDLAAIEAAIQDNTVAIYFETPTNPLLQITDVRAVTVLARRHHLKTIVDNTFATPYNQR 84
1.106           MQDLEAVENAIQDNTVAVYFETPTNPLLQIADIKAIADIAKKHGIKTIVDNTFATPYNQQ 85
1.648           MQDLEAVENAIQDNTVAVYFETPTNPLLQIADIKAIADIAKKHGIKTIVDNTFATPYNQQ 84
1.976           --------------------ETPTTPLLQIADIKAIADIAKKHGVKTIVDNTFATPYNQQ 40
Lb. reuteri     MQDLEAVENAIQDNTVAVYFETPTNPLLQIADIKAIADIAKKHGVKTIVDNTFATPYNQQ 180
B. subtilis     TSSREEVEKAIRPNTKAIYIETPTNPLLKITDLTLMADIAKKAGVLLIVDNTFNTPYFQQ 180
                            ****.***:::*:  :: :*:   :  ::**** *** *.

1.890           PLTLGADIVVHSATKYLGGHSDVVGGIAVTNDEEIAEQLAFIQNSIGAVLGPDDSWLLMR 144
1.1206          PLTLGVDIVVHSATKYLGGHSDVVAGLAVTNDEKIAEDLAFLQNSIGSVLGPDDSWLLMR 144
1.397           PLTLGADVVVHSATKYLAGHSDVVAGIAVTNDAALADRLAFLQNSLGATLGPDDSWLVQR 144
1.106           PLTLGADIVVHSATKYLGGHSDVVAGLAVTNDDEIAEQLAFLQNSIGSTLGPDDSWLLQR 145
1.648           PLTLGADIVVHSATKYLGGHSDVVAGLAVTNDDEIAEQLAFLQNSIGSTLGPDDSWLLQR 144
1.976           PLTLGADIVVHSATKYLGGHSDVVAGLAVTNDDEIAEQLAFLQNSIGSTLGPDDSWLLQR 100
Lb. reuteri     PLTLGADIVVHSATKYLGGHSDVVAGLAVTNDDEIAEQLAFLQNSIGSTLGPDDSWLLQR 240
B. subtilis     PLTLGADIVLHSATKYLGGHSDVVGGLVVTASKELGEELHFVQNSTGGVLGPQDSWLLMR 240
                *****.*:*:*******.******.*:.** .  :.: * *:*** *..***:****: *

1.890           GIKTLGARMRIHHENTAAVIELLEKDPRVARVLYPGLPDFPGHDIAAKQMDHFGAMVSFE 204
1.1206          GIKTLGARMRIHHENAKLVYDYLENNDKVAKIYYPGNPESVGYDIAKKQMRGFGAMISFE 204
1.397           GIKTLAARMRIHEENAAAVVAFLQSDAHVAKIYYPVLPDFPGHAVAAKQTRGFGAMIAFE 204
1.106           GMKTLGARMRVHQENANEVINFLQNDDHIGKIYYPGLKDFPGHEVAAKQMRNFGAMISFE 205
1.648           GMKTLGARMRVHQENANEVINFLQNDDHIGKIYYPGLKDFPGHEVAAKQMRNFGAMISFE 204
1.976           GMKTLGARMRVHQENANEVINFLQNDDHIGKIYYPGLKDFPGHEVAAKQMRNFGAMXXFX 160
Lb. reuteri     GMKTLGARMRVHQENANEVINFLQNDDHIGKIYYPGLKDFPGHEVAAKQMRNFGAMISFE 300
B. subtilis     GIKTLGLRMEAIDQNARKIASFLENHPAVQTLYYPGSSNHPGHELAKTQGAGFGGMISFD 300
                *:***. **.  .:*:  :   *:..  :  : **   :  *: :* .*   **.*  *

1.890           LQTGLSAKKFVESLQVITLAEXLGGX---------------------------------- 230
1.1206          LNEGLDAKKFVESLQLIDLAEXLGGX---------------------------------- 230
1.397           LRAGLDVKKFVEHLQLIDLAEXLGGX---------------------------------- 230
1.106           LMDGLDAKKFVESLQLIDLAEXLGGIX--------------------------------- 232
1.648           LKDGLDAKKFVESLQLIDLAEXLGGX---------------------------------- 230
1.976           ------------------------------------------------------------
Lb. reuteri     LKDSLDAKKFVESLQLIDLAESLGGIESLIEVPAVMTHGSIPREIRLENGIKDELIRLSV 360
B. subtilis     IGSEERVDAFLGNLKLFTIAESLGAVESLISVPARMTHASIPRERRLELGITDGLIRISV 360
```

Figure 27. Alignment of the amino acid sequences derived from the putative *cxl* genes from *L. reuteri* (TMW 1.106 and 1.976), *L. panis* (TMW 1.648), *L. fermentum* (TMW 1. 890), *L. mindensis* (TMW 1.1206) and *L. pontis* (TMW 1.397). Reference sequences from Cbl (*L. reuteri*, accession number AJ293860) and Cgl (*B. subtilis*, accession number U93874) are included. Distinguishing between Cbl and Cgl based on amino acid sequences is not possible. Amino acids with grey background are catalytic residues, the threonine residue printed in white with black background is involved in cofactor binding.

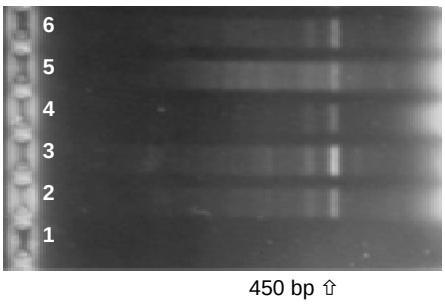

Figure 28. Agarosegel with PCR products obtained with *cxl*-primers and cDNA as template. The cDNA was obtained from mRNA isolated from *cxl* positive and negative strains grown in medium. Lane 1: *L. sanfransiscensis* TMW 1.52 (*cxl* negative), Lane 2-7: *cxl* positive strains: *L. reuteri* TMW 1.106 (lane 2), *L. panis* TMW 1.648 (lane 3), *L. fermentum* TMW 1. 890 (lane 4), *L. mindensis* TMW 1.1206 (lane 5), and *L. pontis* TMW 1.397 (lane 6).

3.8.2 Utilization of cyst(e)ine and methionine by lactic acid bacteria in dough

In table 15, the utilization of cystine and cysteine (abbreviated cyst(e)ine) by lactobacilli during growth in sourdough is shown. This table is a summary of the results that are shown in figure 6 and was made in order to facilitate the consideration whether or not there is a correlation between *cxl* expression and utilization of sulfur-containing amino acids during sourdough fermentation. As compared to a chemically acidified dough, none of the selected strains significantly changed methionine levels as compared to a chemically acidified dough (figure 6), but all cystathionine lyase positive strains lowered the cyst(e)ine levels (figure 6, table 15). However, cystathionine lyase negative strains also lowered the cyst(e)ine levels. Only *L. sanfranciscensis* TMW1.52, which is one of the four cystathionine lyase –negative strains, did not utilize cyst(e)ine, therefore, no clear correlation between cystathionine lyase activity and utilization of sulfur-containing amino acids was observed.

Table 15. Utilization of cysteine and cystine (cyst(e)ine) by lactobacilli in wheat sourdough

Species	TMW-strain -#	*cxl*	Cyst(e)ine levels [a)]
L. sanfranciscensis	1.52	-	100%
L. sanfranciscensis	1.949	-	21%
L. pontis	1.397	+	1%
L. plantarum	1.460	-	3%
L. mindensis	1.1206	+	7%
L. reuteri	1.106	+	9%
L. reuteri	1.976	+	29%
L. fermentum	1.890	+	27%
L. panis	1.648	+	17%
L. brevis	1.465	-	2%

[a)]: Levels of cysteine and cystine relative to those levels in chemically acidified doughs (0.97 mmol / kg). Sourdoughs were fermented for 96h at 30ºC.

3.9 Glutamine deamidation by lactic acid bacteria

3.9.1 Glutamine and glutamate levels during sourdough fermentation

Literature indicates that lactobacilli have the ability to convert glutamine into the taste enhancer glutamate. Sourdoughs were fermented with several sourdough starters and glutamine and glutamate levels were measured. A fungal protease was added to the dough in order to achieve similar proteolytic activity in the doughs. The results are presented in table 16. The glutamine and glutamate levels in the chemically acidified dough were 4.7 and 0.7 mmol / kg dough. In the chemically acidified dough, the percentage of glutamate in the glutamine / glutamate (Glx) fraction was 12 %. In all microbially fermented doughs, the percentage of E was increased 2.5-6-fold, depending on the strain. This indicates that lactic acid bacteria actively deamidate glutamine during sourdough fermentation.

Table 16. Deamidation of glutamine during sourdough fermentation. Presented are the relative amounts of glutamate in the glutamine / glutamate-fraction (Glu/(Glx)) after 24 hours of sourdough fermentation in the presence of a fungal protease. The results are mean values for at least 2 separate fermentations

Strain	TMW#	% Glu in Glx
Chemically acidified	-	12 ±4
L. sanfranciscensis	1.53	62 ±6
L. reuteri	1.106	28 ±1
L. plantarum	1.468	25 ± 5
L. sakei	1.22	40 ± 0
L. perolens	1.501	61 ± 1
L. fermentum	1.890	79 ±3
L. frumenti	1.103	77 ±7
W. confusa	1.928	76 ±4

3.9.2 Role of glutamate in bread flavor

Since lactobacilli convert glutamine into glutamate during sourdough fermentation, it was studied in a triangular test whether the produced levels of glutamate have an impact on the bread taste. All strains produced glutamate during sourdough fermentation, therefore, the reference bread was a bread based on chemically acidified dough whereas the sample breads contained additional glutamate (final concentrations: 21, 47 and 78 mmol / kg sourdough). Three pieces of bread were offered to the assessors, 2 pieces of reference bread and one piece of sample bread. The panelists were asked to eat the bread and identify the differing bread. The results are shown in table 17. Nine out of ten assessors correctly assigned the 47 mmol / kg flour glutamate containing bread as the differing bread and five out of ten assessors were able to discern the 21 mmol / kg flour glutamate containing bread from the reference bread. Thus, the addition of 78 mmol glutamate / kg sourdough significantly ($\alpha = 0.001$, [120]) influences bread taste.

Table 17. Influence of glutamate on bread flavor. Bread crumbs based on chemically acidified preferments were offered to the panelists. Reference preferments did not contain glutamate, whereas different concentrations of glutamate were added to the sample preferments.

Glutamate concentration (mmol / kg sourdough) [a]	Number of panelists that identified the differing sample [b]	Significance [c]
78	9	$\alpha = 0.001$
47	9	$\alpha = 0.001$
21	5	-

[a]: sourdough was added to the bread dough at a 25% level
[b]:10 panelists were questioned
[c]: calculated according to [120]

3.9.3 Glutamine and gliadin deamidation activity in lactobacilli

The increased glutamate levels in microbially fermented sourdough indicate glutaminase activity. However, it is not clear whether glutamine or glutaminyl residues are the preferred substrate: are the proteins first hydrolyzed to amino acids and than deamidated, or are the glutaminyl residues in the protein fraction first deamidated and are the proteins afterwards hydrolyzed by the proteases? Hence, buffer fermentations were performed with either glutamine or gliadin as substrate for deamidation. *L. sanfranciscensis* TMW 1.53 and *L. reuteri* TMW1.106 were used as starter organisms. Glutamine was added to the buffer, and the liberation of glutamate by the resting lactobacilli was monitored. If gliadin is used as a substrate, the only way of monitoring deamidation is by measuring the ammonium release. The results are shown in figure 29A and 29B respectively. Resting cells of *L. reuteri* and *L. sanfranciscensis* both deamidate the single amino acid glutamine (figure 29A). During buffer fermentations, lactobacilli released circa 0.5 mM ammonium (figure 29) from glutamine and gliadins. Prolonged fermentations did not increase glutamate or ammonium levels further (data not shown).

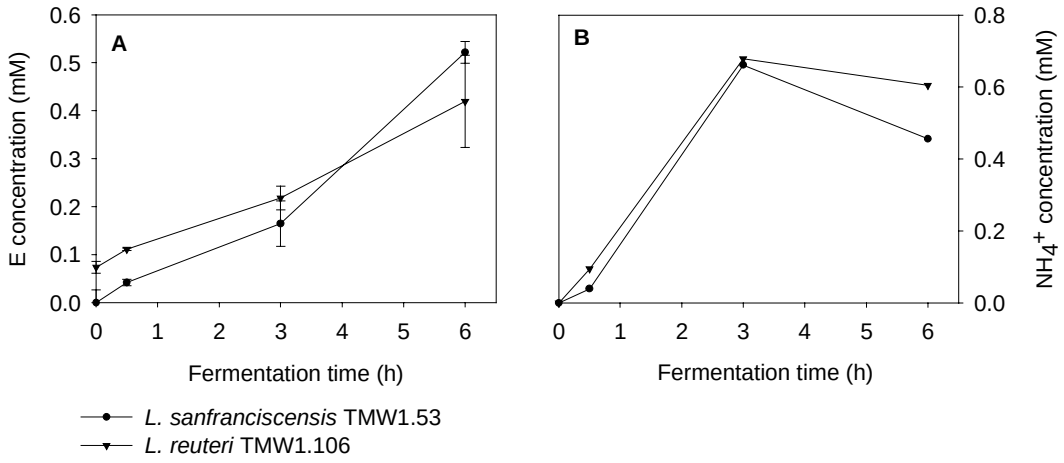

Figure 29. Deamidation activity of lactobacilli during buffer fermentation. Panel A: Glu levels in a Gln containing buffer fermented with resting cells. Presented results are mean values and standard deviations of 2 separate fermentations. Panel B. Ammonium liberation by resting cells in a gliadin-containing buffer.

3.9.4 Deamidation of α2 (58-88) gliadin peptide

In order to investigate whether the release of ammonium during buffer fermentation in the presence of gliadins can be explained by deamidation activity of the substrate, the substrate was replaced by a defined peptide (kindly provided by Dr. Herbert Wieser, Deutsche Forschungsanstalt für Lebensmittelchemie, Garching, Germany) with the following amino acid sequence:

LQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF

This amino acid sequence corresponds with residue 58-88 of α-2 gliadin and is known to evoke celiac disease. ([139]; AJ133612). Buffer fermentations were performed using high cell densities and high

peptide concentrations. Proteolysis was prevented by using whole cells, since the maximum peptide size transported into the cell by the oligopeptide transport system reported thusfar is 18 [76]. In addition, the peptide was chemically deamidated under acidic conditions. This caused a shift in the elution time on RP-HPLC; the deamidation products eluted after the untreated peptide (data not shown), which is in accordance with the results obtained by Wieser [140]. In figure 30, the RP-HPLC chromatograms of the peptide before and after treatment with resting lactobacilli are shown.

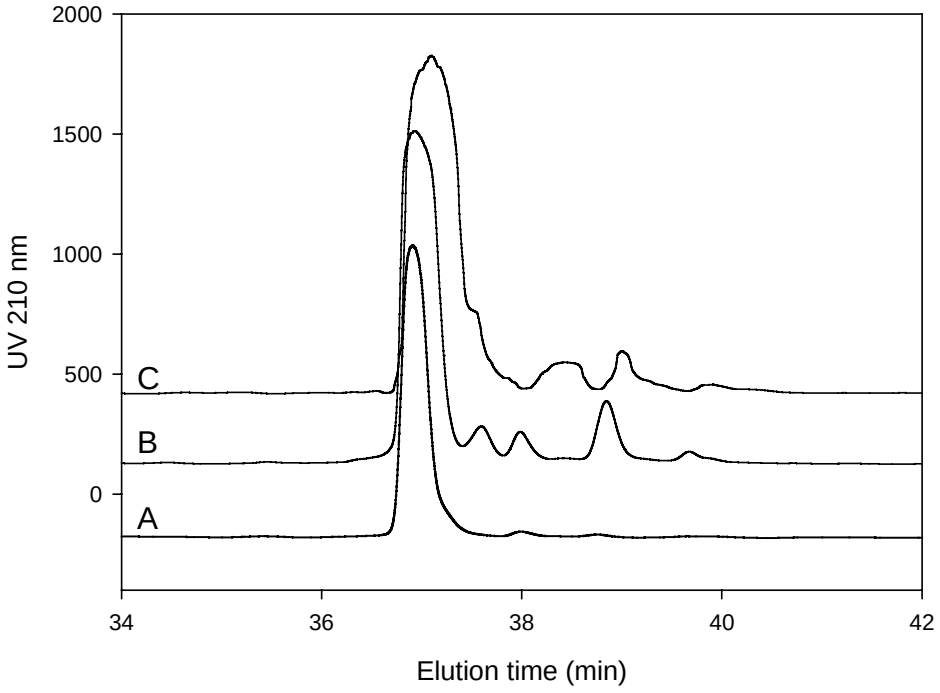

Figure 30. Screening for deamidation of α-2 gliadin (58-88) peptide RP-HPLC chromatograms of untreated synthetic α-2 gliadin (58-88) peptide, 10 mg / ml (A) and after 6 hours buffer fermentation (initial peptide concentration 10 mg / mL) with *L. sanfranciscensis* TMW1.53 (B) and *L. reuteri* TMW1.106 (C) Chromatograms are offset by 300 AU.

Upon incubation with either *L. sanfranciscensis* TMW1.53 or *L. reuteri* TMW1.106, peaks emerge after the main peptide peak, indicating deamidation. In order to exclude the possibility that this was caused by proteolysis, the mass-spectrum of the fermented and unfermented peptide were assessed. The mass-spectrum of the untreated peptide is shown in figure 31. The scattered molecular weight of the synthetic peptide, probably caused by the natural abundance of C^{13}, makes it impossible to detect the deamidation of Q-residues. The mass spectrum of the lactobacilli-treated peptide was acquired as well, and close investigation of the baseline (figure 32a,b) indicates that no proteolysis occurred. On the first sight, there seems to be a shift in molecular weight of the peptide in figure 32 A and B (3696 vs. 3712). This can be explained by the occurrence of sodium and potassium adducts. The difference in molecular weight between figure 32A and B equals the difference in relative atomic mass of Na and K.

Since extraordinary high cell densities were necessary for the effect observed in figure 30, it was assumed that the responsible enzyme is an intracellular enzyme and that the activity of the resting cell

should be attributed to lysed cells. However, when the cells were lysed before incubation, no dramatic increase in activity was observed (data not shown). Thus, the assumption that intracellular enzymes are responsible for the observed effects, was not verified.

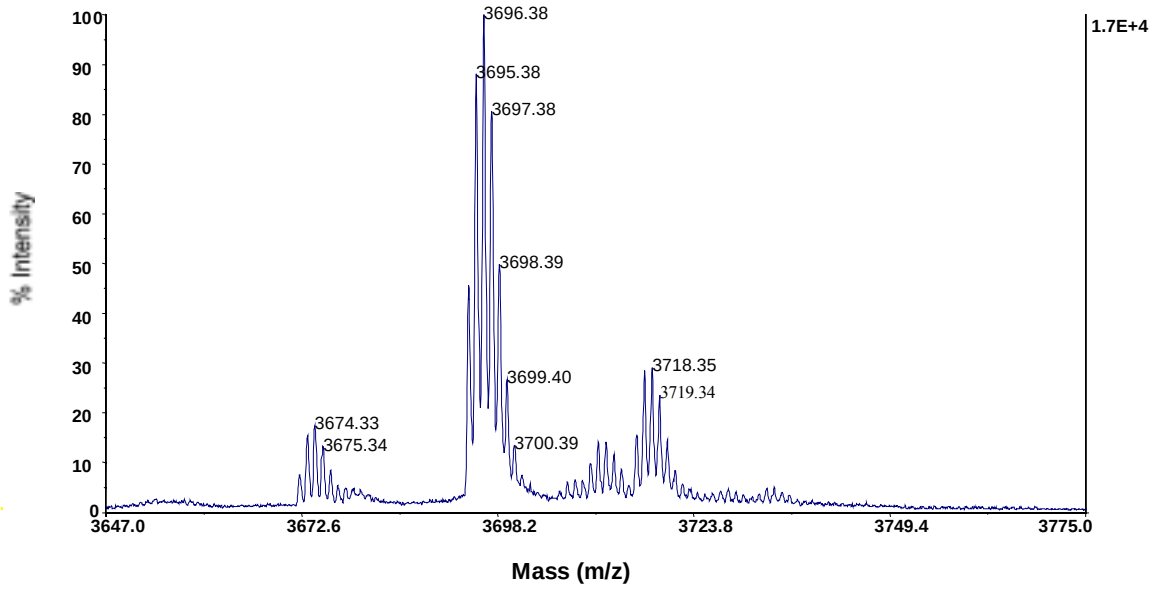

Figure 31. Maldi-ToF mass spectrum of untreated α2 (58-88) gliadin.
Data acquired by 'Zentrallabor für Proteinanalytik, LMU, München, Germany.

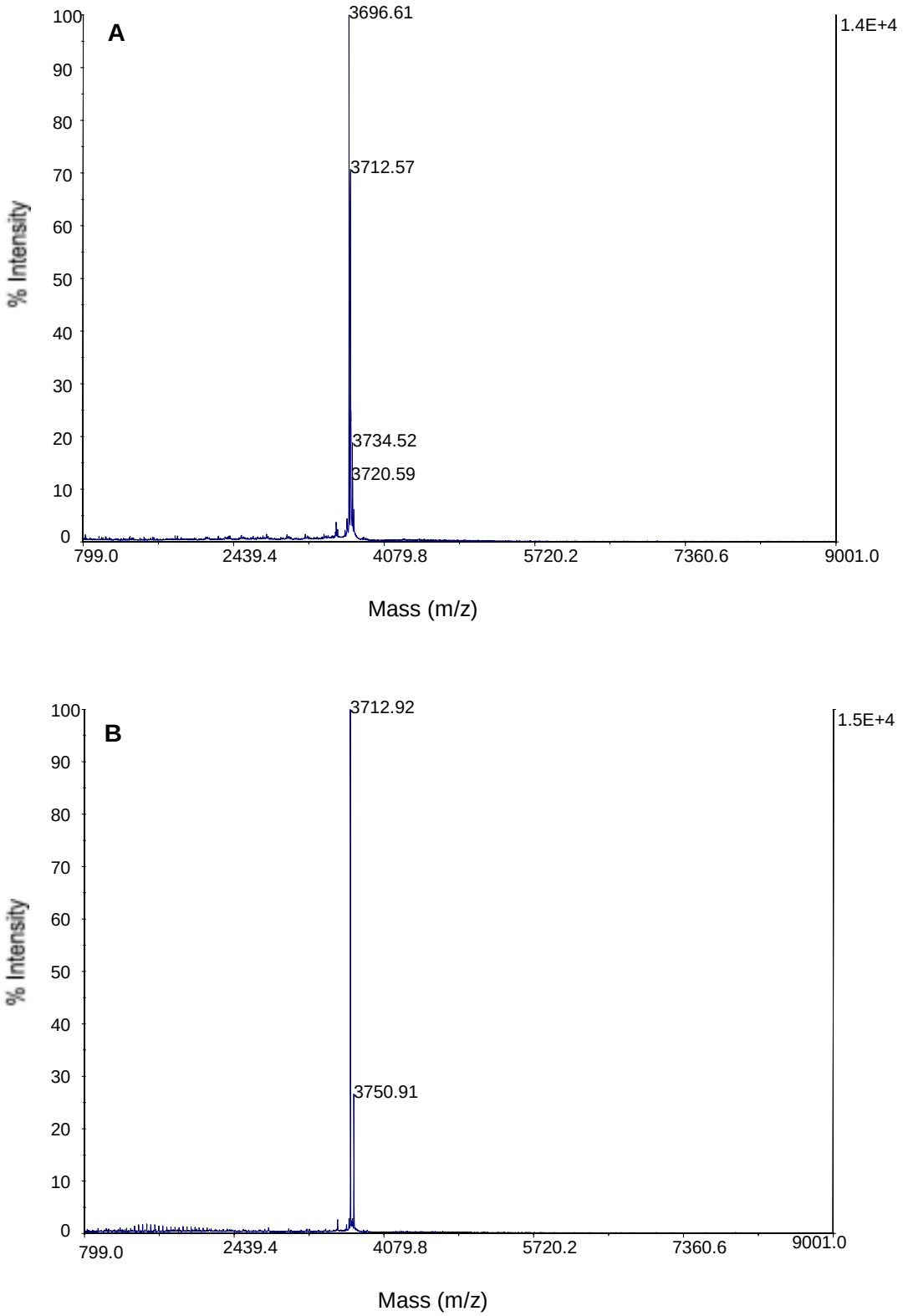

Figure 32. Maldi-ToF mass spectra of α2 (58-88) gliadin aftera 6 hour buffer fermentation with resting cells of *L. sanfranciscensis* TMW1.53 (**panel A**) or *L. reuteri* TMW1.106 (**panel B**).

3.9.5 Glutaminase activity in lactobacilli

In order to facilitate further experiments, the optimum pH and temperature for glutamine deamidation by crude cell extract of *L. reuteri* TMW 1.106 were roughly determined (figure 33) by measuring the ammonium liberation from glutamine. When glutamate was used as a substrate, no NH_4^+ was released (data not shown); thus, the amino group attached to the α-C atom was not enzymatically removed by lactobacilli. The optimum pH and temperature are pH 7 and 37°C, respectively. Asparagine is also a suitable substrate, but is less abundant in wheat protein and therefore irrelevant. Using gliadins instead of glutamine as a substrate, the optimal conditions were the same as for glutamine: pH 7, 37°C.

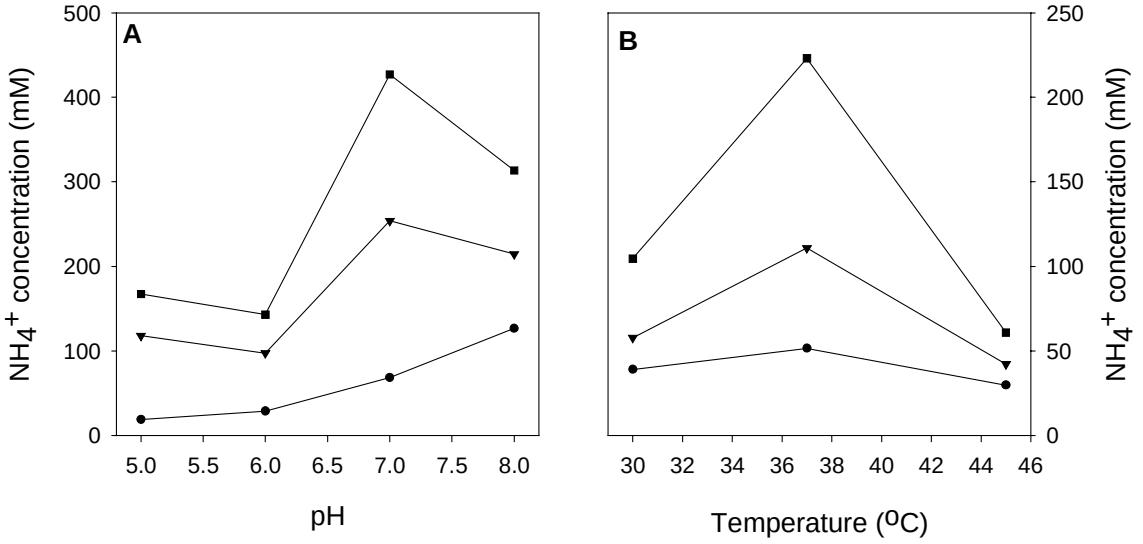

Figure 33. Biochemical characterization of glutamine deamidation by crude cell extract of *L. reuteri* TMW1.106. **Panel A**: Ammonia levels in the reaction after 0.5h (circles), 3h (triangles) and 6h (squares) incubation at 30°C at different pH-values. **Panel B**: Ammonia levels in the reaction after 0.5h, 3h and 6h incubation at pH 7 at different temperatures.

Te attempts to purify the responsible enzyme were not successful. Alternatively, cells of *L. sanfranciscensis* were fractionated, in order to localize the glutaminase activity. The activity and specific activity of the different cell fractions are shown in table 18. Although carry-over of cytoplasm into the membrane fraction and the other way around cannot be avoided, table 18 strongly suggests that the glutaminase activity is localized in the cytoplasm.

Table 18. Localization of glutaminase activity in *L. sanfranciscensis* DSM20451^T. Over-night grown cell were fractioned and the obtained fractions were analyzed with respect to glutamine deamidation activity and protein content.

Cell fraction	Protein content (mg / mL)	Glutaminase activity (mU[a)] / mL)	Specific glutaminase activity (U / mg)
Supernatant	4.3×10^{-3}	not detected	
Cell wall	0.47	8.96	0.05
Cell membrane	0.38	3.34	0.11
Cytoplasm	0.90	2.65	0.34

[a)]: 1 enzyme unit was defined as he amount of enzyme that catalyzes the deamidation of 1 micromole glutamine per minute

Glutaminase (EC 3.5.1.2) is amongst lactobacilli only found in *L. johnsonii* NCC 533 (NC005362) and *L. gasseri* ATCC33323 (draft of the complete genome sequence available at http://img.jgi.doe.gov). Based on these sequences, two pairs of degenerated primers were designed and used for glutaminase screening in the 8 lactobacilli also used for the dough experiment (section 3.8.1), but no amplificates were obtained by PCR (data not shown). Glutamine synthetase (synonym: glutamate ammonia ligase, EC 6.3.1.2) catalyzes the following reaction:

$$ADP + P + glutamine \rightleftharpoons ATP + glutamate + NH_3$$

In *B. subtilis*, this enzyme is responsible for the glutamine biosynthesis. GlnA isolated from *Pyrococcus* sp. is able to catalyze both reactions [141]. Activity of this enzyme during sourdough fermentation can be expected for several reasons. First, the reaction yields energy. The peptide transporters and intracellular peptidases are active and sufficient glutamine should be available, because wheat protein has a high glutamine content. Furthermore, the produced NH_3 might be advantageous because it has a positive effect on acidic stress and competitiveness. Genes probably encoding glutamine synthetase are found on the genomes of several lactobacilli: *L. plantarum* WCFS1 (AL935256), *L. sakei* (NC007576), *L. delbrueckii* (D10020, [142]), *L. casei* ATCC334 (draft of the complete genome on-line available at http://img.jgi.doe.gov), *L. acidophilus* NCFM (CP000033) and *L. rhamnosus* (AJ224996, [143]). Based on these sequences, degenerated primers were constructed and used for screening. The results are shown in figure 34. It can clearly be seen from that although all lactobacilli actively converted glutamine into glutamate during sourdough fermentation, not all lactobacilli harbored a gene encoding glnA.

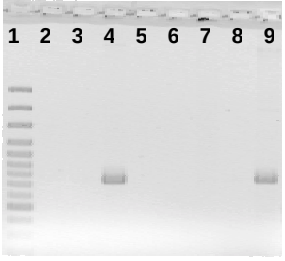

Figure 34. Distribution of *glnA* amongst lactbacilli. Agarose gel with PCR products obtained with *glnA*-primers and chromosomal DNA as template. Lane 1: Base pair ladder, lane 2: *L. sakei* TMW1.22, lane 3 *L. sanfranciscensis* DSM20451^T, lane 4: *L .frumenti* TMW1.103, lane 5: *L. reuteri* TMW1.106, lane 6: *L. plantarum* TMW1.468, lane 7: *L. perolens* TMW1.501, lane 8: *L. fermentum* TMW1.890, lane 9: *W. confusa* TWM1.928.

4 Discussion

The superior aroma of sourdough breads is caused by the microbial activity during fermentation and does not rely on acidification only (for a recent review, see [13]). Microbially fermented sourdoughs were reported to contain higher levels of aroma compounds than chemically acidified doughs [14]. Levels of flavor volatiles in sourdough breads depend on the used sourdough starter [15;16;17;18;19] and the formation of these odorants during sourdough fermentation by lactic acid bacteria and yeasts differs significantly among and within the species [17]. The over-all aim of the work was to screen for and characterize metabolic activities of lactobacilli that are relevant for the sourdough bread aroma. The metabolic potential of a broad variety of strains was investigated: fermented doughs were characterized using instrumental and sensory analyses. *L. perolens* was found to produce diacetyl (odor quality: buttery) and this metabolic feature and its impact on the bread aroma were studied in detail, since in pastry and white wheat rolls, the consumer might consider a slight buttery odor pleasant.

Two groups of substances greatly affect the bread crumb aroma: unsaturated aldehydes originating from lipid oxidation and metabolites originating from amino acid metabolism (table 1). Therefore, the focus of the work was on the influence of lactobacilli on the levels of these compounds. It was shown that heterofermentative lactobacilli and *S. cerevisiae* reduce the levels of odorants that originate from lipid oxidation. *S. cerevisiae* efficiently reduced these compounds, but also enhanced their formation during prolonged sourdough fermentation, as did the homofermentative organism *L. sakei*.

The liberation of amino acids from the dough proteins and their uptake into the cell by *L. sanfranciscensis* DSM20451^T was studied. It was shown that lactobacilli contribute only indirectly to dough proteolysis. The influence of co-substrate availability on metabolite formation from phenylalanine by *L. plantarum* TMW1.468 and *L. sanfranciscensis* DSM20451^T was studied using dipeptides instead of the single amino acid, because dipeptides were more efficiently converted into metabolites than single amino acids. Bottlenecks for amino acid conversion in lactobacilli were identified and strategies for the elimination of these bottle necks were developed. It was shown that lactobacilli have different features with respect to the metabolism of sulfur-containing amino acids. Furthermore, it was discovered that lactobacilli produce glutamate *in situ* during sourdough fermentation.

4.1 Biodiversity

Twenty-four lactic acid bacteria strains were used as starters for sourdough, and the sourdoughs were analyzed with respect to the levels of single amino acids and organic acids (table 7). The fermentation quotients were clearly higher than the optimal 2-2.7 (table 7). This is probably caused by the long fermentation times. Acetate formation is depending on the availability of electron acceptors. As soon as these are depleted, only lactate is produced, which causes an increase in fermentation quotient.

Thus, considerable amounts of acetate are produced, although the fermentation quotient is high. Acids are only volatile when they are protonated. Due to the high levels of lactate produced, the dough pH was rather low (ca. 3.6), and a considerable portion of the acetic acid (pK_a 4.7) was protonated. Therefore, the produced levels of acetic acid might well influence bread aroma. Furthermore, all strains of *L. sanfranciscensis, L. pontis*, and *L. reuteri* that were known to express glycosyltransferases (see table 2) produced higher levels of acetate (15 – 20 mmol / kg) compared to strains without glycosyltransferase activity, which substantiates for a large number of strains the previous observation in *L. sanfranciscensis* TMW1.392 that glycosyltransferases are the only enzymes in sourdough lactobacilli carrying out sucrose hydrolysis [144].

Differences concerning the amino acid contents were observed between microbially fermented doughs and chemically acidified doughs (figure 6). These differences can be attributed to direct or indirect contribution of the lactobacilli to dough proteolysis (section 4.4) and to amino acid consumption be these organisms. All strains capable of ornithine formation from arginine quantitatively converted arginine during fermentation, as was previously observed in fermentations with *L. pontis* TMW 1.397 [7;118]. Furthermore, with the exception of the reference strain *L. sanfranciscensis* TMW 1.52, all strains quantitatively consumed cysteine or cystine. For two strains, *L. sanfranciscensis* TMW 1.52 and *L. perolens* TMW 1.500, increased levels of leucine and phenylalanine were observed when compared to chemically acidified doughs. This phenomenon was observed only once: it was not observed in subsequent experiments with different flours (data not shown). It is therefore questionable whether or not these increased levels reflect the ability of these strains to liberate leucine and phenylalanine from proteins or peptides.

The addition of α-ketoglutarate or leucine did not result in an increased amino acid conversion (section 3.1) by *L. perolens*, *L. sakei* and *L. sanfranciscensis*, as was observed for cheese and sausage [87;88;89;90;114;132]. The difference between cheese and sausage ripening and sourdough fermentation is mainly that the first two processes take months and the cells may die and lyse during the ripening process, whereas sourdough fermentation lasts for one week maximum, and the cells do not lyse. Thus, in contrast to cheese ad sausage, the relevant enzymes are not free in the environment and hence regulation mechanisms and limited substrate availability due to low transport efficiency inhibit metabolite formation.

4.2 Citrate metabolism of *L. perolens*

The addition of 15 mM citrate to mMRS caused an increase in acetate levels from 1.3 mM (data not shown) to 19.6 mM, an enhanced production of α-acetolactate and in an increased growth rate. Fifteen mM of the 18.3 mM additional acetate can be explained by citrate lyase activity, which cleaves citrate into acetate and oxaloacetate (figure 3). The other 3.3 mM could originate from pyruvate that is converted via acetyl-P to acetate. This yields additional ATP and may explain the enhanced growth rate upon the citrate addition. The ability to metabolize citrate positively influenced the growth rate of

Lactococcus lactis in milk [145]. Since 15 mM citrate yields 15 mM additional pyruvate, only 20% is used for energy production. The other 80% are at least partially used by α-acetolactate synthase. *L. plantarum* strains metabolized citrate into acetate with a molar ratio 1:1.7 [146], which is much higher than *L. perolens*. This might be due to low expression levels of the relevant enzymes in *L. perolens*. A maximum of 6 mM α-acetolactate can be produced in the laboratory medium, but quantification was not possible. The fact that α-acetolactate levels were not decreasing with increasing fermentation time (figure 7,8) indicates that the α-acetolactate decarboxylase activity is zero or close to zero. *L. perolens* might not harbor α-acetolactate decarboxylase, or it might be repressed by the branched-chain amino acids that are present in the medium and dough [36].

The addition of citrate to a sourdough fermented with *L. perolens* significantly changed the odor of the preferment and the odor of the bread crumbs based on the preferment (table 8). An untrained panel perceived a buttermilk-yogurt note in the preferments, but not in the breads. The buttermilk-yogurt note in the preferment will most likely be correlated to α-acetolactate formation by *L. perolens* that is oxidized to diacetyl. The bread crumbs were described as more acidic, and only one assessor mentioned buttermilk-yogurt. Either the buttery note of diacetyl is 'overruled' by other notes that are present in the bread crumb, or diacetyl is lost during bread making. The diacetyl might be lost during baking; the boiling point of diacetyl is 88°C, and, the crumb temperature exceeds 90°C during baking and subsequent cooling for approximately 25 minutes [147]. Therefore, diacetyl could evaporate through pores in the bread crust. A second possibility is that the diacetyl present in the dough may be reduced to 2,3-butandiole by the baker's yeast. The reduction of diacetyl (responsible for an off-flavor in beer) by *S. cerevisiae* enzymes is very important in the brewing process. Diacetyl is formed during the beer fermentation, and slowly reduced during beer maturation [148]. The reduction of diacetyl is even the rate limiting step during beer maturation, which indicates that yeast has only a low preference for diacetyl reduction as compared to other aldehydes, like (E)-2-nonenal and (E,E)-2,4-decadienal. The NADH-dependent diacetyl reductase activities of lager- and ale-brewing yeasts was shown to be strain-dependent [149;150]. Furthermore, the conditions during beer fermentation and maturation are completely different from sourdough conditions; gene expression and enzyme activity could be completely different as well. It is therefore not possible to predict the reduction of diacetyl in dough by baker's yeast.

The enhanced levels of acetate due to citrate lyase activity probably caused the acidic note. The use of heterofermentative organisms is more efficient way to achieve an acidic odor than the use of homofermentative, citrate-utilizing strains. In some applications, like pastry, the acidic aroma of the acetate might be undesired. However, other ingredients could mask the acidic taste of acetate and the acidic aroma could be suppressed by increased bread pH, since only the undissociated form is volatile. Alternatively, other compounds that cause a pyruvate excess could be used. For example, sourdough with high dough yield could be aerated or stirred, causing higher oxygen concentrations in the dough. In the presence of oxygen, NADH oxidase uses oxygen as electron acceptor for NAD regeneration.

This makes cofactor recycling by lactate dehydrogenase unnecessary and a pyruvate surplus is the consequence. Measures have to be taken in order to prevent diacetyl volatilization. In this work, no increase in α-acetolactate was measured when *L. perolens* was allowed to grow under aerobic conditions. This can be explained by the fact that in the presence of oxygen, α-acetolactate is oxidized to diacetyl, which is volatile and might be lost. Instead of air-borne oxygen, other electron acceptors might be suitable compounds for creating an intracellular pyruvate excess as well.

4.3 Reduction of (E)-2-nonenal and (E,E)-2,4-decadienal during sourdough fermentation

4.3.1 Levels of (E,E)-2,4-decadienal and (E)-2-nonenal in wheat sourdoughs

In the chemically acidified dough, (E,E)-2,4-decadienal levels increased during the first 24 hours of fermentation (figure 9). (E,E)-2,4-Decadienal is a secondary product of the oxidation of linoleic acid. Five to ten minutes after dough mixing, 45% of the flour lipids had been oxidized and longer mixing periods did not enhance lipid oxidation further [151]. (E,E)-2,4-Decadienal levels increased during the first 24 hours of fermentation (figure 9). This ongoing oxidation of fatty acids may be attributable to the liberation of unsaturated fatty acids from tri / di / mono acylglycerols by cereal or microbial enzymes during sourdough fermentation [152;153]. Furthermore, flour lipids that are entrapped in the gluten network may be released concomitantly with the solubilization of the gluten during wheat sourdough fermentation [52]. In doughs fermented with *S. cerevisiae* or *L. sakei*, higher (E,E)-2,4-decadienal contents were reached after 5 hours than in the chemically acidified dough. *S. cerevisiae* and *L. sakei* produce H_2O_2 in the presence of oxygen [57;154], which suggests NADH oxidase activity (catalyzed reaction: $NADH + H^+ + O_2 \rightarrow H_2O_2$). H_2O_2 production by *S. cerevisiae* was specifically shown in wheat doughs [155], which could promote the oxidation of linoleic acid to (E,E)-2,4-decadienal. After 24 hours, the (E,E)-2,4-decadienal concentration decreased in the chemically acidified dough, indicating chemical or enzymatic decomposition of (E,E)-2,4-decadienal.

4.3.2 Metabolism of (E,E)-2,4-decadienal and (E)-2-nonenal in *L. sakei*, *S. cerevisiae* and *L. sanfranciscensis*

Doughs were spiked with either (E)-2-nonenal or (E,E)-2,4-decadienal. After fermentation, the concentrations of the aldehydes and corresponding alcohols were analyzed, in order to study which microorganism was able to reduce these compounds (figure 10). A metabolic turnover of the higher aldehydes by *L. sakei* was not detected. The results indicate that *L. sanfranciscensis* reduced the aldehyde moiety, since the corresponding alcohol was recovered. In contrast, in doughs fermented with *S. cerevisiae*, aldehyde levels decreased, but the corresponding alcohol was not recovered. For *S. cerevisiae* and *L. sanfranciscensis*, the metabolic pathways for unsaturated aldehyde reduction were studied more in detail, because. *S. cerevisiae* reduced both the aldehyde moiety and the double bond, indicating that it harbors both alcohol dehydrogenase and a CH=CH reducing enzyme. Kinetic studies were performed in order to investigate whether the conversion of (E)-nonenal to nonanal is directed

via (E)-2-nonenol or via nonanal (pathways are depicted in figure 11). In figure 12, it is shown that nonanal levels remained below detection limit throughout the fermentation whereas the unsaturated alcohol (E)-2-nonenol first accumulated, and than disappeared again. If pathway 1 in figure 11 is preferred over pathway 2, the reduction of nonanal to nonanol is so fast, that nonanal does not accumulate. This implies that the reduction of (E)-2-nonenal to nonanal is the rate limiting step in this pathway, and (E)-2-nonenal levels remain high and are available for the reduction of the aldehyde moiety, causing a rise in (E)-2-nonenol, which can indeed be seen in figure 12. The second reaction in pathway 2, the hydrogenation of the double bond, is the rate limiting reaction in pathway 2. (E)-2-Nonenal was almost qualitatively converted into (E)-2-nonenol, as can be seen in figure 12. Thus, the reduction of the double bond was slow whereas the reduction of the aldehyde moiety was fast and therefore, pathway 2 was preferred over pathway 1 (figure 11) by *S. cerevisiae* growing in sourdough. Based on these results, it can not be excluded that a small portion of (E)-2-nonenal is converted to nonanol via nonanal. Wanner and Tressl [156] incubated crude cell extract from *S. cerevisiae* with (E)-2-octenal and NADH and quantified the metabolites. The fact that traces of the saturated aldehyde were found suggested that, in contrast to the results presented here, the double bond was reduced first by an enone reductase, and then the aldehyde by an alcohol dehydrogenase. Two enone reductases were purified from *S. cerevisiae* that did reduce α,β-unsaturated double bonds of carboxylic acids, and alcohols were not tested [156]. The preference for the double bond or aldehyde moiety depends on enzyme specificity, but also on enzyme activity in the cell.

Using (E)-2-nonenal as a substrate, *L. sanfranciscensis* reduced the aldehyde to the corresponding alcohol (E)-2-nonenol. NADH-dependent alcohol dehydrogenase activity with hexanal and nonanal as substrates was observed in crude cellular extracts of *L. sanfranciscensis* [157], the corresponding enzyme in *L. brevis* has been characterized on the genetical and biochemical level [158]. Likewise, *L. reuteri* rapidly reduced (E,E)-2,4-decadienal levels in dough (figure 9). The reduction of aldehydes is coupled to the oxidation of NADH to NAD, enabling heterofermentative strains to produce acetic acid instead of ethanol from acetyl-P, yielding extra ATP (figure 2).

S. cerevisiae also reduced unsaturated aldehydes, although slower. During biomass production in yeasts, an excess of NADH is produced. This NADH can be oxidized in many reactions, for example during degradation of amino acids [159], or during the production of glycerol from glucose (figure 7). The latter reaction is energetically disadvantageous, because the glucose cannot be used for energy production. Baker's yeast produces glycerol during dough fermentation [160]. Hence, reduction of unsaturated aldehydes in the dough might enable the yeast to use glucose for glycolysis rather than for NAD regeneration and might thus be energetically beneficial.

4.3.3 Relevance of (E,E)-2,4-decadienal and (E)-2-nonenal metabolism for bread flavor

(E,E)-2,4-Decadienal is a key aroma compound in the wheat bread crumb. The odor threshold of (E,E)-2,4-decadienal in wheat starch is 0.0027 mg / kg, whereas experience showed that the odor

threshold of (E,E)-2,4-decadienol is 100-1000 times lower (personal communication Dr. M. Czerny, Deutsche Forschungsanstalt für Lebensmittelchemie). Consequently, (E,E)-2,4-decadienol has not been reported not contribute to bread flavor. In sourdoughs fermented with various microorganisms, the levels in (E,E)-2,4-decadienal ranged from 0.17 mg / kg for *L. sanfranciscensis* to 1.5 mg / kg dough for *L. sakei* after 3 hours of fermentation. The levels of hexanal in wheat bread crumb were essentially unchanged compared to the levels in the corresponding dough [13], therefore, (E,E)-2,4-decadienal levels can be expected to remain nearly unchanged during baking as well. The large differences of (E,E)-2,4-decadienal concentrations that were observed in the various sourdoughs dough are thus likely to result in corresponding concentration differences in the bread crumb, and in a significantly contribution to bread flavor.

4.4 Influence of redox-reactions catalyzed by homo- and heterofermentative lactobacilli on gluten polymerization in wheat sourdoughs

The GMP contributes to dough and bread properties in several ways. One the one hand, the GMP contributes greatly to the bread volume. On the other hand, when the GMP is solubilized, proteolysis can take place and the resulting amino acids are precursors for odorants: they can be converted to odorants either via microbial conversion or via chemical reactions that take place during baking. It was therefore objective to investigate how lactobacilli are influencing the oxidative cross-linking of the glutenins. Experiments aimed to elucidate whether (i) lactobacilli affect the level of SH groups in wheat dough and wheat protein, and (ii) display enzyme activity relevant for SS-SH interchange during growth in sourdough.

4.4.1 Influence of lactobacilli on the thiol content in wheat dough and wheat protein

As visualized in figure 35, proteolytic degradation of gluten proteins in wheat doughs is limited by the enzyme activity, and by the limited solubility of the substrates, mainly gluten proteins. Accordingly, acidification to activate gluten-associated aspartic proteases, and the addition of reducing agents to solubilize gluten proteins enhance proteolysis in wheat doughs [7;51;55]. The combined addition of an industrial protease and a reducing agent to chemically acidified doughs enabled a virtually quantitative proteolytic degradation of wheat proteins within four days of fermentation (this work). A similar synergistic effect of industrial protease and microbially fermentation was previously reported in sourdoughs fermented with the heterofermentative *L. pontis* [52]. This synergy between protease addition and lactic fermentation also occurred in doughs fermented with the obligatory heterofermentative *L. sanfranciscensis*, but not with facultative heterofermentative organisms such as *L. perolens* and *L. sakei* (this work), and none of the strains used exhibits appreciable proteolytic activity [53]. Therefore, it was tested whether heterofermentative lactobacilli increase the solubility of gluten proteins through reduction of intermolecular disulfide bonds.

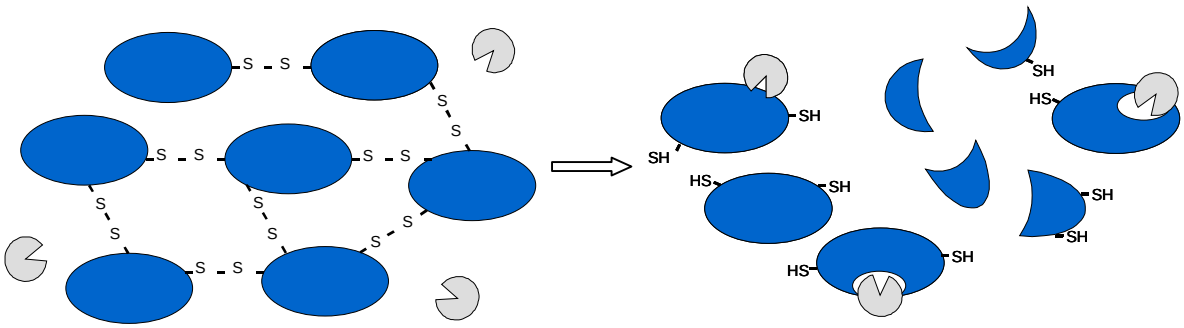

Figure 35. GMP solubilization and degradation. Proteases can not degrade the gluten macropolymer, unless it is depolymerized by reduction of the SS bonds. Whereas formation and reduction of those bonds is reversible, proteolysis is irreversible.

Evidence for the formation of SH groups in heterofermentative lactic metabolism was obtained by the determination of SH levels in chemically acidified doughs and sourdoughs. In *L. sanfranciscensis* sourdoughs, the SH-levels remained essentially constant throughout 24h of fermentation, an effect that was also observed in chemically acidified doughs to which 10 µM GSH g^{-1} dough were added. In contrast, free SH levels decreased in chemically acidified doughs and *L. perolens* sourdoughs. The decrease in free SH groups in a dough fermented with *L. sakei* was even more pronounced as in chemically acidified dough. *L. sakei* is able to produce H_2O_2 [57], that could promote the oxidative cross-linking in a manner similar to ascorbic acid or potassium bromate [161].

The total amount of glutathione and related SH compounds in their free, oxidized and protein-bound forms in type 550 wheat flours is about 0.5 µmol g^{-1} flour [46;162], corresponding to about 0.25 µmol g^{-1} dough. After 24 hours, the total SH concentration in *L. sanfranciscensis* sourdoughs and chemically acidified doughs were 3.4 and 2.4 µmol g^{-1} dough (type 550 flour as well), respectively (this work, figure 15), indicating that disulfide bonds in SDS-soluble proteins were reduced by *L. sanfranciscensis* in addition to low-molecular weight SH compounds. An effect of *L. sanfranciscensis* on SH groups in the gliadin fraction was validated by direct measurement of SH groups in the propanol-soluble proteins according to Antes and Wieser [127]. The propanol-soluble protein fraction comprises the majority of glutenin proteins after chemically acidified or sourdough fermentations [52;53]. In agreement with previous investigations, the amount of propanol-soluble proteins was approximately the same in chemically acidified doughs and *L. sanfranciscensis* sourdoughs. However, proteins from *L. sanfranciscensis* sourdoughs were more efficiently labeled with DACM, indicating a higher level of SH groups in gluten proteins extracted from sourdoughs compared to gluten proteins of unfermented doughs or chemically acidified doughs.

4.4.2 Glutathione dehydrogenase activity of *L. sanfranciscensis* DSM20451^T during growth in sourdough

The intermolecular cross-linking of glutenin proteins is dependent on the presence or absence of low-molecular weight SHs that are oxidized through enzymatic activities from wheat with oxygen as

terminal electron acceptor [49]. During dough mixing, GSH is oxidized to GSSG by glutathione dehydrogenase with L-threo-dehydroascorbic acid as an oxidant [46]. Subsequently, dehydroascorbic acid is oxidized to ascorbic acid with oxygen as an oxidant by ascorbic acid oxidase. Both GSH and GSSG react with SH groups in the protein fraction [46;161]. High levels of GSH or related SH compounds (Cysteine, Glu-Cys and Cys-Gly) that are not oxidized during dough mixing interfere with the cross-linking of glutenin proteins through disulfide bonds [46;49]. A direct effect of *L. sanfranciscensis* on disulfide bonds in gluten proteins is unlikely because these are inaccessible to intracellular or cell-wall bound enzymes of *L. sanfranciscensis*. More likely, *L. sanfranciscensis* enzymes will react with the low-molecular weight SH components involved in this cascade. Several strains of *L. sanfranciscensis*, including strain *L. sanfranciscensis* TMW1.52 used in this work, exhibit NADH-dependent glutathione reductase activity [58]. In this work, it was demonstrated that *L. sanfranciscensis* converts extracellular GSSG to GSH during buffer fermentation and that the gene coding for the responsible enzyme, glutathione reductase, was expressed by *L. sanfranciscensis* during sourdough fermentation. Detection of gene expression alone does not give any information about the actual enzyme activity. As described in section 3.4.4, *L. sanfranciscensis* was able to convert extracellular GSSG to GSH during buffer fermentation, indicating that under these conditions, glutathione reductase activity is present. Furthermore, thiol levels in dough fermented with a Δ*gshR-L. sanfranciscensis* strain, resembled thiol levels of chemically acidified doughs (personal communication Prof. M. Gänzle, University of Alberta, Canada). Taken together, these data suggest that the glutathione reductase of *L. sanfranciscensis* contributes to the effects of sourdough fermentation on SH levels in sourdough according to the scheme proposed in figure 36. In baking industry, ascorbic acid is used to prevent dough softening. The improver reaction of ascorbic acid is based on reaction by which most of the GSH is incorporated in the glutenin before it could cleave the already existing intermolecular disulfide bonds in the GMP (figure 36A). Microbial glutathione reductase activity however, could release GSH from GSSG, causing a softening of the dough (figure 36B). Reduced cofactors are continuously formed during fermentation in the heterolactic metabolism of glucose (figure 1), whereas the supply of oxygen or other oxidants in doughs that restore intermolecular disulfide bonds is limited.

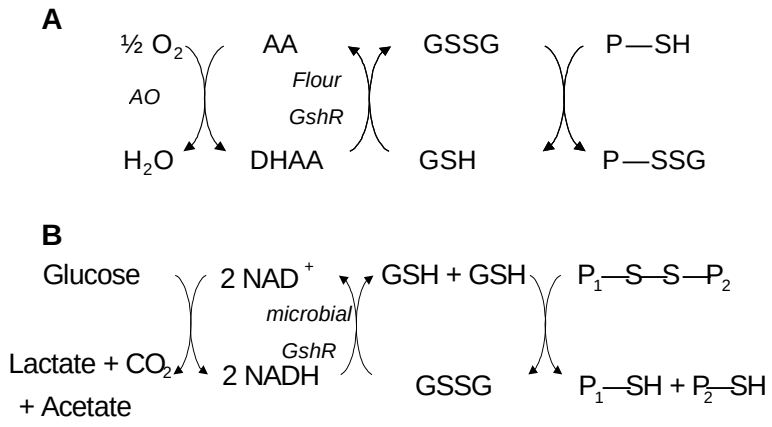

Figure 36. Proposed reactions involved in thiol-disulfide interchange reactions in wheat sourdoughs. **A**. Oxidation of glutathione with oxygen as terminal electron acceptor by wheat enzymes, drawing according to the more detailed presentation of Hahn and Grosch [47]. **B**. Proposed interference of *L. sanfranciscensis* with gluten cross-linking based on its GshR activity. AA, ascorbic acid, DHAA, dehydroascorbic acid, AO, ascorbic acid oxidase, GSSG and GSH, oxidized and reduced glutathione, respectively, GshR, glutathione reductase, P, protein.

In industrial practice, sourdough that may or may not contain viable microorganisms is added to wheat doughs up to a 10 % level. Sourdough addition to bread dough at a 20% level decreased the gluten quality in the resulting bread dough [50]. Proteolytic degradation of glutenin subunits irreversibly deteriorates the GMP, but gluten depolymerization based on redox-reactions can be reversed through the appropriate choice of additives at the dough stage. Because homo- and heterofermentative lactobacilli have opposite effects on gluten cross-linking, the choice of starter cultures provides an additional means to control the quantity and the quality of the GMP in wheat doughs. The GMP is a major determinant of dough rheology and bread texture of wheat breads produced by straight dough processes. Therefore, it is reasonable to assume that these parameters are affected by redox-reactions of lactobacilli in industrial applications. These results may thus enable an improved control of the effects of sourdough on gluten quality.

4.5 Proteolytic activity of *L. sanfranciscensis* DSM20451^T

The results presented in section 3.5 failed to provide evidence for the presence of Prt-like cell-wall associated protease in the *L. sanfranciscensis* type strain. First, *L. sanfranciscensis* was not able to grow on casein as sole nitrogen source. Previously, a screening of 108 sourdough isolates has shown that only five exhibited proteolytic activity, indicating proteolytic activity is strain-dependent and that the lack of cell-wall associated proteinases is the rule rather than the exception in sourdough lactic acid bacteria [70]. Second, proteolytic activity in chemically acidified doughs and sourdoughs was inhibited upon the addition of an aspartic protease inhibitor, indicating that cereal proteinases are responsible for the first step in gluten degradation. Aspartic protease inhibitors but not serine proteinase inhibitors were previously reported to inhibit proteolytic activities in sourdough extracts

[51]. Third, screening the genome of *L. sanfranciscensis* DSM20451^T did not provide evidence for a *prt*-like proteinase.

Gobbetti et al. [71] purified and characterized a 57 kDa cell envelope serine proteinase from *L. sanfranciscensis* CB1 grown in a gluten containing medium. Prt-proteinases from lactic acid bacteria have a relative molecular weight of 135-145 [63], but proteinases with 45 and 180 kDa are also reported in the literature [74]. Poquet et al. [163] noticed that the *prt*-negative *L. lactis* MG1363 has extracellular proteolytic activity, that was attributed to HrtA, the sole cell surface housekeeping serine protease involved in protein maturation and turnover. Additionally, HtrA is a key factor to the response to several stress conditions in *L. lactis* [164] and in *L. helveticus* [165]. HtrA from *E. coli* was characterized as a serine protease with a relative molecular weight of 48 which is, under physiological conditions, active as homo-dodecamer [166;167;168]. However, the monomers displayed endopeptidolytic activity towards casein [167]. The *L. sanfranciscensis* DSM20451^T genome was screened with degenerated primers targeting *htrA*, but no PCR-amplificate was obtained (data not shown). This unfortunately neither proofs nor excludes that *L. sanfranciscensis* harbors a functional HrtA.

4.6 Peptide utilization and regulation of peptide transport and hydrolysis by *L. sanfranciscensis* DSM20451^T

Analysis of peptide- and amino acid levels in sourdough has shown that *L. sanfranciscensis* LTH2581 uses peptides to meet its nitrogen demand during growth in sourdough [7;52] and *L. sanfranciscensis* ATTC27653 requires peptides for optimal growth [72]. In this work, it was observed that chemically defined peptides but not amino acids support growth of *L. sanfranciscensis* LTH 2581 on N-limited mMRS (data not shown). This indicates that *L. sanfranciscensis* is auxotroph for at least one amino acid that must be transported into the cell as part of peptides. Genes encoding two peptide transport systems, *dtpT* and *opp,* and five peptidases, *pepC*, *pepX*, *pepR, pepN* and *PepT*, were found on the genome of *L. sanfranciscensis* DSM20451^T. An aminopeptidase [71] and X-prolyl dipeptidyl aminopeptidase were purified from *L. sanfranciscensis* CB1 cell extract and were assigned PepN and PepX [169].

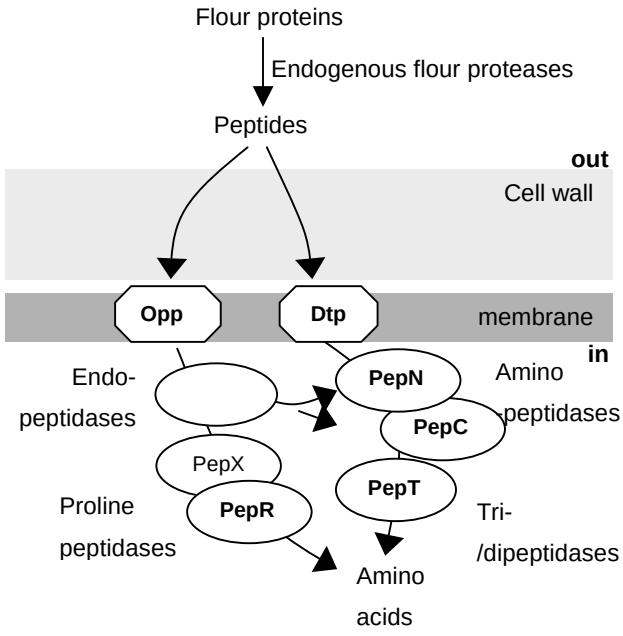

Figure 37. Schematic overview of the peptide utilization of *L. sanfranciscensis* DSM20451^T during sourdough fermentation retrieved by a genome sampling approach. Opp, oligopeptide transport system. DtpT: Di- and tripeptide transport system. Proteins printed in bold refer to genes transcribed during sourdough fermentation.

A schematic overview of the thusfar known genes involved in peptide utilization of *L. sanfranciscensis* DSM20451^T and their expression during sourdough fermentation is given in figure 37. It was shown that Opp, DtpT, PepT, PepC, PepR and PepN are expressed during sourdough fermentation (section 3.6.2). The addition of peptides to dough resulted in reduced transcription of both *dtpT* and *opp-pepN* in exponentially growing cells. During the stationary growth phase, *dtpT* and *opp-pepN* transcription were reduced regardless of the addition of peptides. Thus, the high expression of peptide transporters in exponentially growing cells is attributable to the limited peptide supply in dough rather than increased nitrogen requirements during exponential growth. Because amino acids and peptides accumulate during fermentation, externally added peptides no longer affect the expression of peptide transporters during stationary phase. The regulation of the PepT peptidase upon the addition of peptides was much less pronounced. Peptide availability is therefore a limiting factor in amino acid metabolism.

The putative proteins identified in *L. sanfranciscensis* are highly similar to the corresponding proteins in *L. lactis*. In *L. lactis*, the proteolytic system consists of three peptide transporters and 17 intracellular peptidases [170]. Because only 20% genome coverage was achieved with the genome sampling approach [134], additional peptidases and / or peptide transport systems are likely to be present in *L. sanfranciscensis*. The findings concerning the organization and regulation of the proteolytic system of *L. sanfranciscensis* only partially overlap with *L. lactis* [170]. In both organisms, Opp expression was strongly reduced by addition of peptides and PepT expression was reduced two-

to five-fold. DtpT was regulated in response to the peptide supply in *L. sanfranciscensis* but not in *L. lactis*.

The levels of amino nitrogen were higher in *L. sanfranciscensis* sourdoughs than in chemically acidified dough. This difference was more pronounced when peptides were added to the dough. Similar observations have been made by Di Cagno et al. [42;171], who reported that sourdough fermentation results in a more complete degradation of peptides compared to an chemically acidified dough. This implies that the difference between *L. sanfranciscensis* fermented doughs and chemically acidified doughs depends on a synergistic effect between flour proteinases and the peptide metabolism of *L. sanfranciscensis*. The peptides are taken up by the cells, hydrolyzed and a part of the amino acids is used for further metabolism. Remaining amino acids that are not needed may be excreted into the dough.

4.7 Phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM20451^T and *Lactobacillus plantarum* TMW1.468

Metabolites from branched-chain and aromatic amino acids are particularly odor active. As mentioned in the introduction, the metabolic pathways and regulation mechanisms involved in the conversion of these amino acids are very similar [60]. Thus, general interpretations may be derived from experiments with one of those amino acids. Phenylalanine was used as model amino acid, since phenyl groups are easy to detect and the metabolites of phenylalanine are both odor active and antifungal. The formation of PLA from phenylalanine has been described as strain dependent [98;99;100]. Three independent explanations can be given for this fact [172]: (i) the genes coding for the key-enzymes involved in flavor formation are only harbored by a fraction of the strains of a species, (ii) the occurrence of different variants of enzymes among strains, (iii) the expression of many relevant enzymes is strongly subject to regulation. Hence, rational prediction and optimization of amino acid metabolism was not possible and an empirical approach was needed. Therefore, specific properties of transport and conversion rates in two different strains were studied, in order to get an insight in which factors may be of importance for metabolite formation. In this work, distinct differences were observed in PLA formation and amino acid metabolism and its regulation in *L. sanfranciscensis* DSM20451^T and *L. plantarum* TMW1.468. These differences between the two strains are partially explained by species specific metabolic differences, however, as only one strain was considered for each of the species, strain specific differences may additionally influence the effect of co-factors on amino acid metabolism.

4.7.1 Transport efficiency limits phenylalanine conversion

Low intracellular amino acid levels are reported to limit amino acid metabolism, due to the poor transport of single amino acids; transamination of amino acids is more efficient when cells are permeabilized [173;174] or lysed [175;176]. The peptides used in this study were all quantitatively

hydrolyzed by the intracellular peptidases. Accumulated F was transported out of the cell, either actively or by facilitated diffusion, or it leaves the cell via passive diffusion as was shown for proline [75]. In this work, it was shown that peptides stimulate PLA formation more than single amino acids. One can therefore conclude that transport of single amino acids is not very efficient in lactobacilli, which results in low intracellular amino acid concentrations and poor amino acid conversion. This was overcome when peptides instead of single amino acids are used as a substrate. This was overcome when peptides instead of single amino acids were used as a substrate. In sourdough, however, the increase of PLA production is similar upon the addition of single amino acids and peptides. During sourdough fermentation, the expression of genes involved in peptide transport is lowered, due to the high levels of peptides present in the dough (section 4.5). This could explain why peptide addition does not increase PLA formation during dough fermentation.

4.7.2 Increase of the metabolic flux by enhancement of the transamination rate

Phenylalanine and PLA accumulate during fermentation, whereas the intermediate metabolite phenylpyruvate was not found in the medium. Hence, transaminase activity was lower than peptide transport, peptide hydrolysis and phenyllactate dehydrogenase activity. Although the genes encoding transaminases were expressed in medium (table 14), it was not possible to measure the enzyme activity using biochemical tests as described previously for *L. lactis* [78;86]and *L. helveticus* [177] (data not shown). The transaminase activity was so low, that no reliable results were obtained. This illustrates that gene expression is no measure for enzyme activity.

Transamination is, after transport efficiency, the second bottleneck in PLA formation identified in this work. Transamination is the rate limiting step in the formation of metabolites from amino acids in many other lactobacilli as well. Since transamination can be stimulated using α-ketoglutarate, cofactor availability and not enzyme activity is the limiting factor [87;88;89]. The addition of α-ketoglutarate was sufficient to stimulate PLA formation in *L. plantarum*. PLA formation in *L. sanfranciscensis* was only enhanced if citrate was added as well. In *L. sanfranciscensis* α-ketoglutarate could be replaced by glutamate, which can be explained by the Gdh activity of this strain. In *L. plantarum*, the addition of FE instead of FX to the medium did not result in enhanced PLA levels, which correlates with the absence of Gdh activity. These observations are in accordance with Tanous et al. [92], who reported that Gdh-positive strains degrade amino acids more efficiently in the presence of glutamate as compared to Gdh-negative strains.

A combination of citrate and α-ketoglutarate or glutamate was required to stimulate amino acid metabolism by *L. sanfranciscensis*. These compounds did not enhance transamination by influencing the expression of the transaminase (table 14). This effect has been attributed to the production of additional α-ketoglutarate [93]: Citrate is transformed to oxaloacetate, which serves as an amino acceptor for the deamidation of glutamate resulting in the formation of α-ketoglutarate and aspartate. Generation of additional α-ketoglutarate upon the addition of citrate does not explain the stimulatory

effect observed in this work, because citrate is required for the efficient use of externally added α-ketoglutarate and aspartate levels in sourdough were not elevated, when citrate and α-ketoglutarate were added to the dough (data not shown). The stimulatory effect of citrate can not be attributed to the generation of a proton motive force, because malate metabolism, which has a similar effect on proton export as citrate, failed to increase PLA production (figure 22). However, a combined effect of fructose and citrate on the production of PLA production from FL was observed (figure 22), which links the flux through the transaminase reaction to the availability of oxidized NAD in the Gdh-positive strain *L. sanfranciscensis* DSM20451^T. Therefore, the NAD / NADH ratio in the cell appears to play a significant role in amino acid metabolism. Addition of fructose did not enhance PLA formation if FS was the substrate. Serine could be converted into pyruvate, as has been observed for *L. lactis* [178], this would influence the redox status of the cell as well. Contrary to fructose, the combination of citrate and α-ketoglutarate enhanced PLA formation from both FS and FL, indicating that the synergistic effect does not rely on changes in the NAD / NADH ratio only. In conclusion, the combined stimulatory effect of citrate and α-ketoglutarate cannot be explained by the availability of additional amino acceptors as was speculated by Tanous et al. [93], but changes in the NAD / NADH ratio seem to play a role in amino acid catabolism. Experiments with ^{14}C-labeled substrate could be used to elucidate more precisely, why addition of both citrate and α-ketoglutarate causes an increase in amino acid conversion.

In *L. lactis*, aromatic aminotransferase activity is increased when branched chain amino acids are removed from the environment, which was attributed to transcription regulation by CodY [179]. Both in *B. subtilis* and in *L. lactis*, the transcription repressor CodY senses intracellular levels of branched chain amino acids [95;96]. *L. sanfranciscensis* harbors *codY* and the amino acid sequence of CodY$_{L. sanfranciscensis}$ differs from CodY$_{L. lactis}$ in 1 amino acid (data not shown). Addition of leucine and valine as part of a peptide to the medium did not significantly change PLA formation, nor did the addition of proline. Furthermore, the presence of FL in the medium did not influence *araT1* and *araT2* expression significantly. Thus, CodY regulation is not negatively influencing PLA formation by *L. sanfranciscensis*.

Bottlenecks in the production of aroma-relevant compounds from amino acids by *L. sanfranciscensis* DSM20451^T an *L. plantarum* TMW1.468 were identified and strategies to increase the metabolic flux were found. Distinct differences were observed in metabolite formation from phenylalanine, which can be explained by the different set of enzymes present in both strains and by differences in regulation processes. Amino acid catabolism is no side effect of other enzyme activities, since distinct genes encode the relevant enzymes. To what gain lactobacilli express these genes and catabolize aromatic and branched-chain amino acids remains unknown. Although the intracellular NAD / NADH ratio plays a role in amino acid metabolism, a major energetic advantage of amino acid catabolism does not exist. Lactobacilli seem to be more sensitive to these catabolites than molds are, thus the production of antifungals as main reason for amino acid catabolism can be excluded. As long

as the main motivation of amino acid catabolism is unknown, the control of these processes will be based on empirical developed strategies and probably not optimal.

4.7.3 Formation of PLA during sourdough fermentation

The addition of α-ketoglutarate and citrate influenced PLA formation in dough in the same way as was observed in nitrogen-limited medium. In sourdough, PLA levels are generally higher than in medium. This is attributable to generally higher substrate concentrations in dough resulting in higher final cell densities in sourdough as compared to nitrogen-limited medium. Specifically, peptide concentrations are much higher than in nitrogen-limited medium. This explains the absence of an increase in PLA formation when F was replaced by FS in dough systems because peptide transport systems are down regulated during growth of *L. sanfranciscensis* in sourdough when the peptide availability increases (section 3.6.2).

4.7.4 Antimicrobial activity of PLA

In liquid medium, 45 mM PLA is required in order to inhibit mold growth [180], which is in accordance with the results presented in figure 26. Mold growth was delayed by 5 days in wheat bread produced using sourdough started with a phenyllactic acid producing *L. plantarum* strain in comparison to a bread produced using sourdough started with a non-phenyllactic acid producing *L. brevis* strain [9]. It was anticipated that low levels of PLA inhibit fungal growth due to synergistic interactions with other antifungal compounds [97]. Combined with lactate and acetate, the antifungal activity of PLA was indeed increased (figure 26), but *L. sanfranciscensis* was more sensitive to PLA and lactic acid / acetic acid mixture than the tested molds. *B. subtilis* was more sensitive than *L. sanfranciscensis*. Growth of staphylococci and enterococci was inhibited by PLA [181]. *Lactobacillus sanfranciscensis* hardly grows at PLA levels >2 mM. All attempts to increase PLA production by lactobacilli will fail to cross this barrier, unless a less sensitive strain is found, or fermentation conditions are applied that decrease the antimicrobial activity of PLA (e.g. a buffered system).

The mode of action of PLA was investigated in the gram-positive organism *Listeria monocytogenes*; it causes changes in the bacterial cell (wall) structure and finally cell lysis [181]. In sourdough fermentation, PLA levels did not exceed 1.2 mM. During baking however, water evaporates and is bound to gelatinized starch [182]. Therefore, the water activity decreases and local concentrations of PLA and other organic acids may be higher in the bread. It is therefore possible that even the PLA levels measured here influence mold growth on baked goods. Increased transamination will not only enhance PLA levels, but will also stimulate the degradation of other aromatic amino acids present in the environment, since the aminotransferases have a rather broad substrate specificity [80]. This will cause a rise in other antifungal organic acids such as hydroxyphenyllactic acid, which originates from tyrosine.

4.8 Metabolism of sulfur containing amino acids by lactobacilli

Cystathionine lyase (Cxl) is a key enzyme in the metabolism of sulfur containing amino acids in lactic acid bacteria (figure 4). The degradation of cyst(e)ine in preferments was investigated, as was the distribution of *cxl* amongst lactic acid bacteria. Five of 24 tested strains were found to be *cxl* positive (figure 27). The positive strains were mostly strains that are usually found in industrial sourdoughs that are maintained at elevated temperatures [33]. It cannot be excluded that the degenerated primers failed to anneal to *cxl* in some cases, giving rise to 'false-negatives'. Analysis of the five obtained partial sequences showed that all the amino acid residues essential for catalysis and cofactor binding were conserved. Other amino acid residues involved in cofactor binding and catalysis are located outside the sequenced area [138] and therefore it is impossible to predict whether the transcription results in functional proteins.

Upon the addition of methionine and cysteine, the methanethiol production by *Lactococcus lactis* subsp. *cremoris* decreased, indicating a down-regulation of the involved enzymes [103]. In lactobacilli however, the methionine concentration had no effect on the cystathionine lyase activity [109]. Likewise, expression of *cxl* in medium was observed for all strains that harbored the gene (figure 28). The cystathionine lyase isolated from *L. fermentum* is more active on cystine than on cystathionine [106]. Taken together, these facts indicate that this enzyme fulfills a catabolic function in lactobacilli. The conversion of cystine by cystathionine lyase yields pyruvate (figure 5). Pyruvate is substrate for lactate dehydrogenase, this cofactor regeneration enables heterofermentative species to gain more energy from hexose metabolism, as was mentioned before. On the other hand, in *L. delbrueckii* subsp. *bulgaricus*, cystathionine-β-lyase deficiency correlated with methionine auxotrophy [183]. Furthermore, the cystathionine-γ-lyase operon of *L. fermentum* was shown to play a role in the protection against oxidative stress [184]. It was speculated that cystine is converted into pyruvate by cystathionine lyase, which than non-enzymatically reacts with hydrogen peroxide [185], resulting in water and acetate, as was shown in *L. lactis* [186]. However, this hypothesis assumes a low lactate dehydrogenase activity, resulting in a pyruvate accumulation. This is rather unusual in lactic acid bacteria [187]. The second possibility is that cystathionine lyase activity gives rise to (homo)cysteine (figure 5), which in turn could be oxidatively cross-linked and might serve as oxygen scavengers and protect against oxidative stress. In this work, all strains expressing *cxl* partially or fully consumed the cyst(e)ine available in sourdough. A biosynthetic function of *cxl* seems therefore unlikely. *Cxl*-negative strains also consumed cyst(e)ine and methionine during sourdough fermentation (table 15), indicating that other enzymes play a role in the metabolism of sulfur-containing amino acids as well. Seefeldt and Weimer [109] suggested that other enzymes than cystathionine lyase contribute more to the production of sulfur-containing volatile compounds from amino acids. Based on these results, no clear statement can be made about the role of *cxl* in cyst(e)ine metabolism and its possible influence on dough properties. Therefore, a cystathionine lyase knock-out mutant should be constructed and thoroughly screened.

4.9 Deamidation of glutamine by lactobacilli

In literature, few direct and indirect indications can be found that lactobacilli are able to convert glutamine into the taste enhancer glutamate [104;112;113;114;115;116;117]. Wheat protein is extraordinary rich in glutamine [43]. The percentage of glutamate in the glutamine / glutamate fraction was significantly higher in microbially fermented doughs as compared to a chemically acidified dough (table 16). Thus, lactobacilli are *in situ* producers of glutamate. *Lactobacillus sanfranciscensis* and *L. reuteri* converted glutamine into glutamate during buffer fermentations. Glutamate is commonly used as a taste enhancer in food industry. The addition of 47 mmol glutamate / kg to sourdough significantly ($\alpha = 0.001$, [120]) influences bread taste (section 3.9.2). Whether this is reached during sourdough fermentation or not, depends on the fermentation time and degree of proteolysis. In a normal sourdough that was fermented with *L. sanfranciscensis* for 24 h, the amino acid concentration is about 15 mmol / kg dough (figure 14B, more experimental data are available, but not shown). Assuming a glutamine content of 40%, this will not be enough to influence the bread taste (section 3.9.2). However, after 96h fermentation in the presence of a fungal protease, the amino acid concentration is 95 mmol / kg, which implies almost 40 mmol glutamine / kg dough is released, which is probably enough to influence bread flavor. Since proteolysis is ongoing after microbial growth has ceased, meaning that longer fermentation times cause higher levels of amino acids. If glutaminase activity does correlate with growth neither, sufficient *L. sanfranciscensis* might be able to produce high levels of glutamate that significantly influence bread flavor. Thus, in summary, extensive proteolysis influences bread flavor in several ways: first, the increased amino acid levels cause an increase in Strecker and Maillard reaction products; second, an increased bioconversion of branched-chain and aromatic amino acids results in the accumulation of aroma-active compounds and third, the formation of glutamate from glutamine significantly influences bread flavor. The last two points might even interact: glutamate enhances amino acid conversion in the presence of citrate, as was shown in this work.

In food industry, gluten hydrolysates are commonly used as food additive. Microbially hydrolyzed gluten, which can be labeled ‘natural’ or ‘biological’, has a higher consumer acceptance level compared to chemically hydrolyzed gluten, which must be labeled ‘artificial’. In this thesis, it was shown that wheat protein can be efficiently degraded by lactobacilli and that these organisms accumulate glutamate during growth in sourdough. Lactic acid fermentation based gluten hydrolysis is cheap, and easy to control thanks to the vast experience with lactic acid fermentations, and to the available genomes. Thus, optimized sourdough fermentation could be a biological alternative to produce wheat protein hydrolysates.

Lactobacilli release ammonium from extracted gliadins (figure 29) and if the α2-gliadin (58-88) peptide is treated with lactobacilli, change of hydrophobicity is observed, but no proteolysis occurs (figures 30, 32). This indicates that glutaminyl-residues in peptides can be deamidated by lactobacilli.

The efficiency of glutaminyl-residue deamidation was approximately the same for whole cells and lysed cells, whereas glutamine was much more efficiently deamidated by cell extract as compared to whole cells. Thus, two different enzymes catalyze these two reactions and the enzyme responsible for glutaminyl-residues deamidation might be an extracellular one. Using gliadin as a substrate, ammonium is released (figure 29b). Given that the glutamate content of gliadin is 40%, that the gliadin concentration in the described experiment was 1 mg mL^{-1}, and that the molecular weight of one glutaminyl residue is 128, the glutaminyl concentration can be estimated and is approximately 3 mM During the fermentation, circa 0.5 mM ammonium is released figure 29b). In case of glutamine, the substrate concentration was 20 mM, but glutamate levels measured after buffer fermentation were similar to the ammonium levels measured in the gliadin-containing buffer. This low efficiency of glutamine deamidation can be explained by further utilization of glutamate by the cells or by inhibition of the enzyme reaction by the end product. Ammonium release can also be caused by other metabolic activities. Thus, the ammonium levels measured in the gliadin containing buffer lead to an overestimation of the deamidation activity, whereas the glutamate levels measured in the glutamine containing buffer underestimate the deamidation activity. If α2 (58-88) gliadin is treated with resting lactobacilli, a very high cell density is required for a shift in elution time on RP-HPLC (figure 32) and even in that case the majority of the peptide is not affected, as unchanged elution time would imply. These data hint that glutamine is preferred over glutaminyl-residues for deamidation during buffer fermentation.

Transglutaminase, produced by *Streptoverticillium*, is commonly used to improve the properties of food proteins by cross-linking, but is also reported to deamidate gliadins. Transglutaminase is used in baking industry because it improves the gluten network and consequently the bread volume and crumb properties [188]. On the genome of *L. casei* (draft of the complete genome sequence on-line available at http://img.jgi.doe.gov), a transglutaminase-like domain was found. Peptidoglutaminase, an enzyme that resembles transglutaminase but acts on peptides instead of proteins, is produced by *Bacillus circulans* [189]. Yamaguchi et al. [190] purified and characterized a protein-glutaminase that was free of transglutaminase activity from *Chryseobacterium proteolyticum.* Within the course of this work, it was not possible to couple this metabolic feature to a known enzyme.

A pool of several lactic acid bacteria strains was needed to hydrolyze a 33-mer peptide that contained the same amino acid sequence as the α2-gliadin (58-88) peptide [171;191]. These results do not contradict with the results concerning peptide deamidation presented here, since single strains had no effect [191]. Probably, a mixture of strains is required since some strains possess deamidation activity while other strains provide the peptidolytic enzymes and due to this synergy, the peptide can be hydrolyzed. Both the cell wall fraction and the cytoplasmatic fraction of four selected strains of lactobacilli were able to hydrolyze the α-gliadin (31-43) [42]. Although the α-gliadin (31-43) peptide is proline and glutamine rich as is the α2-gliadin (58-88) peptide, its amino acid sequence, is completely different and it is much smaller. This makes it likely that it shows a different susceptibility

to the relevant enzymes. Deamidation of gliadins might have consequences for persons suffering from celiac disease, a chronic inflammatory intestinal disease, induced by wheat proteins. In susceptible individuals, the immune system is triggered by peptides originating from wheat, rye, barley or oat causing a cascade that leads to disruption of the mucosal structure. T-cells of celiac disease patients recognized the α2-gliadin (62-75) only after deamidation [192]. Since the peptide used in this work includes the α2-gliadin (62-75) sequence, the lactobacilli seem to alter the amino acid sequence of one of the triggers of celiac disease. The exact mechanism of celiac disease is very complex; different peptides are involved in the disease in different manners [193]. Therefore, it is not possible to predict whether the deamidation of gliadins by lactobacilli is beneficial or harmful for patients who suffer from celiac disease.

Thus, it was found that lactobacilli convert glutamine into the flavor enhancer glutamate, but this metabolic activity could not be attributed to a known enzyme and that they probably even are able to deamidate gliadins, although at a lower rate. A substantial part of open reading frames on the available genome sequences from lactic acid bacteria has not been successfully assigned to a protein function yet. In order to assign a function to an open reading frame, the amino acid sequence needs to be homologous to another sequence, obtained from a purified, characterized and sequenced enzyme. As long as the enzyme is not purified, a metabolic activity is not recognized. Until now, glutaminase activity is characterized in only one *Lactobacillus* species [116], but it has neither been purified nor sequenced yet. Since deamidation is poorly investigated in lactic acid bacteria, the responsible enzyme is not known yet and is one of the open reading frames with unknown function, the so-called 'hypothetical proteins'. In order to investigate which enzyme is or which enzymes are responsible for the glutamine deamidation sourdough, it will be necessary to purify the enzyme, characterize it, sequence it and find the corresponding hypothetical protein sequence on the available lactobacilli genomes. Subsequently, the gene can be characterized, the enzyme can be expressed heterologue or a knockout mutant can be constructed in order to obtain more insight in this flavor relevant metabolic feature of lactobacilli.

5 Concluding remarks

The over-all aim of the work was to screen for and to characterize metabolic activities of lactobacilli that are relevant for the sourdough and bread aroma, because the superior aroma of sourdough is caused by the microbial activity during fermentation and does not rely on acidification only. In this work, it was found that lactobacilli increase glutamate levels in the dough, it was studied how amino acids are liberated from the GMP and how these amino acids are internalized and converted by the cells, giving rise to aroma relevant metabolites. Furthermore, the influence of lactobacilli on the off-flavor causing compounds (E)-2-nonenal and (E,E)-2,4-decadienal was studied. It should be stressed here, that the conditions applied during the sourdough fermentation in this work were adapted or simplified sourdough fermentations in order to assess the influence of specific metabolic properties. In most fermentations, commercially available wheat flour was used, but all fermentations were performed with single strains, in order to be able to characterize the metabolic potential of these strains. In industrial applications, mixed starters containing both lactobacilli and yeasts are used. In most sourdough fermentations, the fermentation times were often quite long. This is not uncommon in industrial practice, but these sourdoughs are no end-products as such, but are mixed with other components and are subjected to further processes. Therefore, it is not easy to predict which of the described metabolic activities described in this thesis will finally influence bread flavor. In case of glutamine deamidation, it is very likely that this will influence bread flavor; it is not volatile and even after dough leavening with baker's yeast, glutamate significantly changed bread flavor, as was shown in this work.

This work showed that the intracellular NAD / NADH ratio of starter cultures is of great importance for bread quality (figure 38). Heterofermentative lactobacilli energetically benefit from the reduction of external electron acceptors and lower the redox potential of the dough. Fructose was a well-known external electron acceptor present in flour, its conversion to mannitol accounts approximately for 50% of the acetate production in wheat doughs [144;194]. Additionally, the reduction of oxidized glutathione to monomeric glutathione (section 4.4), and of aldehydes originating from lipid oxidation (section 4.3) contribute to NADH regeneration and hence to acetate formation by *L. sanfranciscensis* in sourdough. Thus, the energetic advantage of NADH regeneration for heterofermentative lactobacilli has a strong impact on wheat bread texture and flavor in addition to acetate levels in dough. Lactobacilli metabolizing glucose via the Embden-Meyerhof pathway do not profit from NADH recycling during growth in sourdough. In contrast, homofermentative lactobacilli promote oxidative processes like gluten cross-linking and lipid oxidation. Furthermore, a pyruvate excess in homofermentative lactobacilli diminishes the need for cofactor recycling and enables these organisms to produce diacetyl (figure 3), which influences the sourdough aroma significantly. In addition, the NAD / NADH ratio in the cell appears to play a significant role in amino acid metabolism (section 4.7.2). It can be expected that other redox dependent reactions in the dough, like gluten cross-linking via dityrosine [44], will also be affected in different ways by homo- and

heterofermentative organisms. Baker's yeast plays a more complex role, since it benefits from the use of electron acceptors, causing a decrease in the redox potential, but on the other hand, it produces H_2O_2, causing a rise in the redox potential. The choice of starter lactobacilli can serve as a tool to influence the levels of aroma relevant aldehydes in other kinds of foods as well.

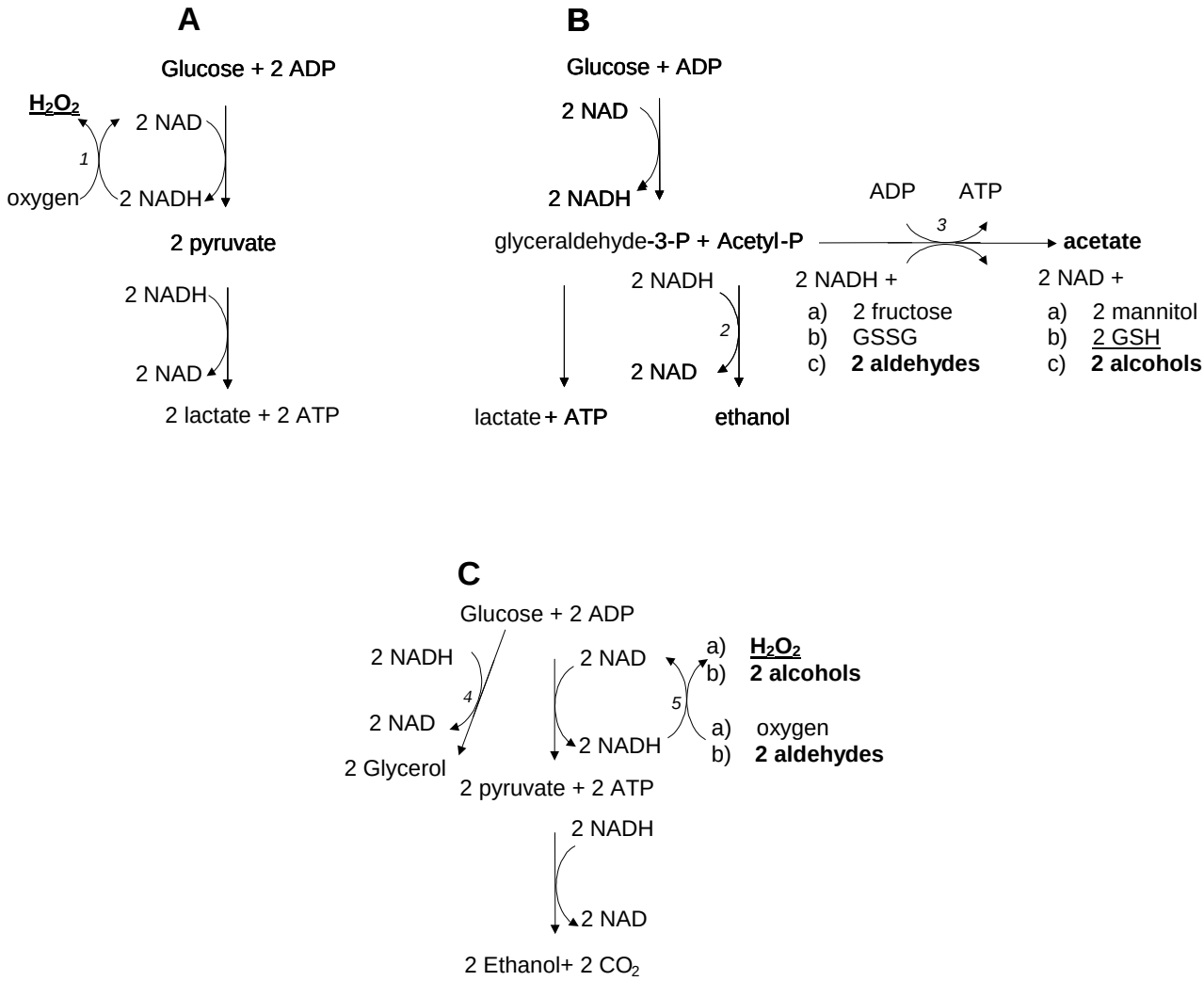

Figure 38 Schematic overview of the relation between sugar catabolism, redox recycling and sourdough properties by homofermentative and facultative heterofermentative (**A**) and obligatory heterofermentative (**B**) lactobacilli and anaerobic fermentation by yeasts (**C**). Metabolism via the Embden-Meyerhof-pathway (**A**) generates 2 mol of ATP per mol glucose. NADH oxidase activity (*1*) generates hydrogen peroxide. Heterofermentative metabolism via the pentose-phosphate pathway (**B**). Per mol glucose, 1 mol of ATP is generated if acetyl-phosphate is used as electron acceptor to produce ethanol (*2*), or 2 mol of ATP in the presence of other electron acceptors, which enable additional ATP-synthesis by conversion of acetyl-phosphate to acetate (*3*). Yeasts convert 1 mol glucose under anaerobic conditions into 2 mol ethanol and 2 mol CO_2, yielding 2mol ATP (**C**). Conversion of glucose to glycerol results in the oxidation of 2 NADH to 2 NAD (*4*). Aldehydes or small amounts of oxygen can be used as electron acceptors, allowing a higher metabolic flux of glucose towards ethanol, CO_2 and ATP. Aroma relevant compounds are printed in bold (section 4.4), hydrogen peroxide enhances lipid oxidation and so the formation of flavor volatiles (section 4.4). Underlined substances influence gluten cross-linking (section 4.3).

The proteolytic system *of L. sanfranciscensis* was characterized and it was found that this organism is devoid of a protease involved in nitrogen supply. Likewise, the *L. plantarum* WCFS 1 genome does not contain *prt*. A comparison of the genomes of *L. johnsonii* LA-1 and *L. plantarum* indicated that their metabolic potential related to peptide uptake, peptide hydrolysis, and amino acid

biosynthesis reflects the adaptation to nutrient rich or poor environments, respectively. The *L. plantarum* harbors no *prt*, and fewer peptidase genes but retained more amino acid biosynthetic capability [136;196]. Biochemical and genetical studies in dairy lactobacilli such as *L. helveticus* and *L. delbrueckii* subsp. *lactis* [66] as well as *L. lactis* [170] demonstrate that these organisms reduce the expression of proteinases if sufficient peptides are present in its environment. Milk is poor in amino acids and peptides, and has low proteolytic activity. Therefore, proteinase activity (Prt) is a prerequisite for growth of lactic acid bacteria in milk. In contrast, meat and cereals contain sufficient amino nitrogen and have a high proteolytic activity and thus support growth of non-proteolytic lactobacilli. In the meat environment, active proteinases are present, whereas the endogenous proteases in wheat flour are not active until the pH is lowered due to the acid production by lactobacilli. Lactobacilli in cereal and meat fermentations both rely on the proteolytic activity of their environment to meet their nitrogen demand.

6 Summary

The aroma of microbially fermented sourdough bread is generally preferred over a chemically acidified bread. The superior aroma of sourdough breads is caused by the microbial activity during fermentation and does not rely on acidification only; lactobacilli are likely to contribute to the levels of odorants and odorant precursors in sourdough and the final bread in several ways. The metabolic potential of a wide variety of lactobacilli during sourdough fermentation was studied in order to screen for interesting metabolic properties. In this work, the production of diacetyl as byproduct of sugar metabolism was studied, as was the reduction of aldehydes originating from lipid oxidation in dough by sourdough microorganisms. The degradation of proteins during dough contributes to the aroma formation in two ways: amino acids are precursors for thermal reactions that greatly influence the crust aroma and they can be metabolized by lactobacilli giving rise to specific metabolites. Therefore, the influence of lactobacilli on gluten solubilization during sourdough fermentation was investigated. The conversion of the released amino acids was studied with respect to aromatic and sulfur containing amino acids, because their metabolites are known to be particularly odorous. Furthermore, the conversion of glutamine into glutamate was studied

This work showed that the intracellular NAD / NADH ratio of starter cultures is of great importance for bread quality. Offering citrate to the homofermentative organism *L. perolens* TMW1.501 resulted in a pyruvate surplus: pyruvate that not originated from sugar catabolism did not have to be used for cofactor regeneration. This pyruvate excess caused diacetyl (odor quality: buttery) accumulation. Furthermore, homofermentative lactobacilli promote oxidative processes like gluten cross-linking and lipid oxidation. Heterofermentative lactobacilli energetically benefit from the reduction of external electron acceptors and lower the redox potential of the dough. The reduction of oxidized glutathione to monomeric glutathione (section 4.4) by *L. sanfranciscensis* caused increased GMP solubilization. The reduction of ldehydes originating from lipid oxidation (section 4.3) by heterofermentative organisms were shown to contribute to NADH regeneration and hence to acetate formation in sourdough. Thus, the energetic advantage of NADH regeneration for heterofermentative lactobacilli has a strong impact on wheat bread texture and flavor in addition to acetate levels in dough. Cofactor regeneration was also shown to play a role in amino acid conversion by lactobacilli.

The fate of (E)-2-nonenal and (E,E)-2,4-decadienal, two key aroma compounds in wheat bread crumb originating from lipid oxidation, was studied during sourdough fermentation. The levels of these unsaturated aldehydes in doughs fermented with homo- and heterofermentative lactobacilli or baker's yeast were investigated, and the underlying metabolic pathways in these microorganisms were identified. The distinct metabolic activities of sourdough microorganisms influence the levels of volatile unsaturated aldehydes in dough. Heterofermentative strains rapidly reduced the concentrations of these aldehydes in dough, whereas *S. cerevisiae* displayed a lower activity. In *L. sanfranciscensis*, the reduction of aldehydes is coupled to the oxidation of NADH to NAD, which enables this heterofermentative strain to produce additional ATP from glucose. *Lactobacillus sakei*, a strain that

produces only lactic acid during sourdough fermentation, did not metabolize (E)-2-nonenal and (E,E)-2,4-decadienal at all. Both *L. sakei* and *S. cerevisiae* appeared to stimulate aldehyde formation during the first hours of dough fermentation, probably due to the hydrogen peroxide production by these two strains. Unsaturated aldehydes originating from lipid oxidation play a dominant role in the aroma of many fermented foods (e.g. fermented sausage, cheese). Thus, the choice of starter lactobacilli can serve as a tool to influence the levels of these aroma relevant compounds in other fermented foods as well.

GMP solubilization during sourdough fermentation has a large impact on the bread quality: intact GMP determines dough rheology and bread volume, whereas the amino acids that are released after degradation play a role in aroma formation. The effect of redox reactions catalyzed by lactobacilli during sourdough fermentations on properties of wheat gluten was studied. First, thiol levels in wheat doughs were determined. Thiol levels in doughs remained high in doughs fermented with *L. sanfranciscensis* or in chemically acidified doughs to which 10 μmol g^{-1} glutathione were added. In chemically acidified doughs without glutathione or sourdoughs fermented with *L. sakei* or *L. perolens*, the thiol levels decreased during fermentation. Second, the amount of thiol-groups in gluten proteins was estimated by RP-HPLC separation of fluorescence-labeled propanol-soluble proteins extracted from wheat doughs. An increase of the SH groups in gluten proteins was observed in protein fractions from sourdoughs fermented with *L. sanfranciscensis* but not from chemically acidified doughs. Finally, it was shown that *L. sanfranciscensis* expresses glutathione-reductase activity during growth in sourdough, and that this activity results in the reduction of extracellular GSSG to GSH. In conclusion, redox reactions catalyzed by lactobacilli determine gluten quality during sourdough fermentations in addition to the pH-dependent activity of cereal proteases. The formation of thiols by *L. sanfranciscensis* interferes with gluten-polymerization. Based on their differences in the central carbon metabolism, homo- or facultative heterofermentative lactobacilli have opposite effects on redox-reactions in wheat sourdoughs.

It was studied whether *L. sanfranciscensis* DSM20541^T directly contributes to proteolytic degradation of the wheat protein fraction as it occurs during dough fermentation. Screening of the *L. sanfranciscensis* DSM20541^T genome with degenerated primers targeting *prt* and analysis of proteolytic activity *in vitro* provided no indication for proteolytic activity. Proteolysis in chemically acidified doughs and sourdoughs fermented with *L. sanfranciscensis* was inhibited upon the addition of an aspartic protease inhibitor. These results indicate that proteolysis was not linked to the presence of *L. sanfranciscensis* DSM20451^T, and that this strain does not harbor a proteinase.

Metabolites from amino acids play a significant role in bread crumb aroma. Therefore, the formation of the metabolites by lactobacilli was studied. The formation of an intracellular amino acid pool was studied on a genetic level in *L. sanfranciscensis* DSM20541^T; genes of *L. sanfranciscensis* DSM20451^T involved in peptide uptake and hydrolysis were identified, and their expression during growth in sourdough was determined. Genes encoding the peptide transport systems Opp and DtpT,

and the intracellular peptidases PepT, PepR, PepC, PepN, and PepX were identified. Both peptide uptake systems and the genes *pepN*, *pepR*, *pepC* and *pepT* were expressed by *L. sanfranciscensis* growing exponentially in sourdough, whereas *pepX* was not transcribed. The regulation of the expression of Opp, DtpT, and PepT during growth of *L. sanfranciscensis* in sourdough was investigated. Expression of Opp and DtpT was reduced approximately 17-fold if the peptide supply in dough was increased. The expression of PepT was found to be dependent on the peptide supply to a lesser extent. Thus, the accumulation of amino nitrogen by *L. sanfranciscensis* in dough is attributable to peptide hydrolysis rather than proteolysis.

Metabolites from branched-chain and aromatic amino acids are particularly aroma active. The metabolic pathways and regulation mechanisms involved in the conversion of these amino acids are very similar. Therefore, only phenylalanine was studied in detail. Rate limiting factors in phenyllactic acid (PLA) formation from phenylalanine by *L. plantarum* TMW1.468 and *L. sanfranciscensis* DSM20451^T were determined. Transport of single amino acids is not efficient in lactobacilli, and only 1% of the offered phenylalanine was converted to PLA. PLA yields were increased 2- to 4-fold when peptides instead of single amino acids were used as a substrate. The accumulation of phenylalanine after peptide addition indicated that, after transport, transamination was the second bottleneck. In *L. plantarum*, PLA yields were increased from 5% to >30%, upon the addition of α-ketoglutarate. In *L. sanfranciscensis*, a combination of both citrate and α-ketoglutarate was required to increase PLA formation. This combined effect of citric acid and α-ketoglutarate can be attributed to changes in the intracellular NAD / NADH ratio.

Wheat protein is extraordinarily rich in glutamine, and glutamate is a flavor enhancer. It was studied whether lactobacilli are able to convert glutamine into glutamate and whether this could influence bread flavor. The percentage of Glu in the Glx fraction was significantly higher in microbially fermented doughs as compared to chemically acidified dough. *Lactobacillus sanfranciscensis* and *L. reuteri* converted glutamine into glutamate during buffer fermentations. Thus, lactobacilli are *in situ* producers of glutamate. This activity was not attributed to a known enzyme. The addition of 9 mmol glutamate / kg to a sourdough significantly influenced bread flavor. Depending on the degree of protein hydrolysis, deamidation activity and sourdough dosage, the estimated maximum level of glutamate in yeast leavened bread dough is 20 mmol / kg dough. It is anticipated that the glutamate produced by lactobacilli during sourdough fermentation influences bread flavor.

Extensive proteolysis influences bread flavor in several ways: first, the increased amino acid levels cause an increase in Strecker and Maillard reaction products; second, an increased bioconversion of branched-chain and aromatic amino acids results in the accumulation of aroma-active compounds and third, the formation of glutamate from glutamine significantly influences bread flavor. The last two points might even interact: glutamate enhances amino acid conversion in the presence of citrate, as was shown in this work.

7 Zusammenfassung

Das Aroma von Sauerteigbroten wird sensorisch positiver bewertet als das von chemisch gesäuerten Broten. Neben der Säurebildung zeigen Laktobazillen eine Vielzahl weiterer Stoffwechselaktivitäten, die das Brotaroma beeinflussen können. Das Ziel dieser Arbeit war es, diese Stoffwechselleistungen zu charakterisieren, um hiermit eine Grundlage für die Entwicklung neuer Sauerteig-Starter und Backmittel auf Sauerteigbasis zu legen. Eine Vielzahl von Laktobazillen-Stämmen wurden auf ihr 'Aromapotential' hin untersucht.

Lactobacillus perolens TMW1.501 produzierte in Sauerteigfermentationen Diacetyl; unter Zugabe von Citrat wurde eine erhöhte Bildung von α-Acetolaktat und eine signifikante Änderung sowohl im Teig- als auch im Brotaroma beobachtet.

(E)-2-Nonenal und (E,E)-2,4-Decadienal sind Schlüsselsubstanzen für das Aroma der Weizenbrotkrume. Der Verbleib dieser ungesättigten Aldehyde in mit homo- und heterofermentativen Laktobazillen und Backhefe fermentierten Teigen wurde untersucht und die zugrundeliegenden Stoffwechselwege in diesen Organismen identifiziert. Die unterschiedlichen Stoffwechselwege dieser Sauerteigmikroorganismen beeinflussen die Konzentration flüchtiger ungesättigter Aldehyde im Teig. Heterofermentative Stämme reduzierten in kurzer Zeit die Konzentration dieser Aldehyde, während *S. cerevisiae* diese bedeutend langsamer reduzierte. In *L. sanfranciscensis* ist die Reduktion von Aldehyden mit der Oxidation von NADH zu NAD verbunden, wodurch dieser heterofermentative Stamm zusätzliches ATP aus Glucose gewinnen kann. *Lactobacillus sakei*, der während der Sauerteigfermentation ausschließlich Laktat bildet, verstoffwechselte (E)-2-Monenal and (E,E)-2,4-Decadienal nicht. Sowohl *L. sakei* als auch *S. cerevisiae* förderten die Aldehydbildung während den ersten Stunden der Sauerteigfermentation; eine mögliche Erklärung hierfür wäre die Produktion von Wasserstoffperoxid und die daraus resultierende gesteigerte Fettoxidation. Ungesättigte Aldehyde aus der Fettoxidation spielen auch in einer Vielzahl anderer fermentierter Lebensmittel eine wichtige Rolle (z.B. Wurst, Käse). Daher kann die Auswahl des richtigen Stamms für Starterkulturen ein Hilfsmittel sein, um die Konzentration dieser Aromastoffe auch in anderen Lebensmitteln zu kontrollieren

Der Einfluss der von Laktobazillen während der Sauerteigfermentation katalysierten Redoxreaktionen auf Weizengluten wurde untersucht. Hierfür wurden die Thiolgehalte in Weizenteigen bestimmt; sie blieben hoch in mit *L. sanfranciscensis* fermentierten oder chemisch gesäuerten und mit 10 μmol g^{-1} Glutathion versetzten Teigen. In chemisch gesäuerten Teigen ohne Glutathion und in mit *L. perolens* oder *L. sakei* fermentierten Sauerteigen nahm die Thiolkonzentration während der Fermentation ab. Weiterhin wurde der Anteil von Thiolgruppen in Kleberproteinen bestimmt, indem fluoreszenzmarkierte Proteine mit Propanol aus Sauerteig extrahiert und mittels RP-HPLC aufgetrennt wurden. Eine Zunahme der SH-Gruppen wurde in den extrahierten Glutenproteinen aus mit *L. sanfranciscensis* fermentierten Sauerteigen beobachtet, nicht aber in den Proteinen chemisch gesäuerter Teige. Des weiteren konnte gezeigt werden, dass *L. sanfranciscensis* während der Sauerteigfermentation eine Glutathionreduktase exprimiert und dass diese Aktivität eine

Umsetzung von GSSG zu GSH zur Folge hat. Abschließend lässt sich feststellen, dass zusätzlich zu der pH-Wert abhängigen Aktivität von endogenen Weizenproteasen durch Laktobazillen katalysierte Redoxreaktionen die Glutenqualität während der Sauerteigfermentation bestimmen. Die Bildung von Thiolen durch *L. sanfranciscensis* beeinflußt die Gluten-Polymerisation. Resultierend aus den Unterschieden im zentralen Kohlenstoffmetabolismus haben homo- und fakulativ heterofermentative Laktobazillen entgegengesetzte Einflüsse auf Redox-Reaktionen in Weizensauerteigen.

Das *L. sanfranciscensis* DSM20451^T-Genom wurde vergeblich mit degenerierten Primern nach *prt* untersucht; eine *in vitro* Analyse der proteolytische Aktivität dieses Organismus ergab keinen Hinweis auf eine proteolytische Aktivität. Die Proteolyse konnte in chemisch gesäuerten Teigen und in mit *L. sanfranciscensis* fermentierten Sauerteigen durch die Zugabe eines Aspartylprotease-Inhibitors nahezu vollständig inhibiert werden. Diese Ergebnisse deuten an, dass die Proteolyse nicht im Zusammenhang mit der Anwesenheit von *L. sanfranciscensis* DSM20451^T steht und dass dieser Organismus nicht über eine Protease verfügt.

Am Peptidtransport und der Peptidhydrolyse beteiligte Gene in *L. sanfranciscensis* DSM20451^T wurden identifiziert und deren Expression während der Sauerteigfermentation wurde untersucht. Es wurden Gene gefunden, die die Transportsysteme Opp und DtpT und die intrazellulären Peptidasen PepT, PepN, PepX, PepC und PepR kodieren. Beide Transportsysteme und *pepN*, *pepR*, *pepC* und *pepT* wurden in der exponentiellen Wachstumsphase während der Sauerteigfermentation exprimiert, *pepX* hingegen nicht. Die Regulierung der Expression von Opp, DtpT und PepT während des Wachstums von *L. sanfranciscensis* in Sauerteig wurde untersucht. Die Expression von Opp und DtpT wurde ungefähr siebzehnfach reduziert, wenn der Peptidgehalt im Teig erhöht wurde. Die Expression von PepT hing weniger stark mit dem Peptidangebot zusammen. Somit ist die Akkumulation von niedermolekularem Aminostickstoff durch *L. sanfranciscensis* in Sauerteig eher der Peptidhydrolyse als der Proteolyse zuzuschreiben.

Limitierende Faktoren in der Phenyllactat-Bildung aus Phenylalanin in *L. plantarum* TMW1.468 und *L. sanfranciscensis* DSM20451^T wurden bestimmt. Der Transport einzelner Aminosäuren in Laktobazillen ist nicht sehr effizient, nur ungefähr 1% des angebotenen Phenylalanins wurde zur Phenyllaktat verstoffwechselt. Die Phenyllaktatausbeute konnte um den Faktor 2 bis 4 erhöht werden, wenn phenylalanin-enthaltende Dipeptide anstelle der einzelne Aminosäure angeboten wurden. Die Akkumulation von Phenylalanin nach Peptidzugabe weist darauf hin, dass nach dem Transport die Transaminierung den zweiten Engpass darstellt. Die Phenyllaktatbildung von *L. plantarum* konnte durch Zugabe von α-Ketoglutarat von 5% auf mehr als 30% gesteigert werden. Um die Phenyllaktatbildung von *L. sanfranciscensis* zu steigern, war die Zugabe von α-Ketoglutarat und Citrat notwendig. Dieser Effekt kann den Veränderungen in der intrazellulären NAD / NADH-Bilanz zugeschrieben werden.

Im Vergleich zu chemisch gesäuerten Teigen ist der Glutamatanteil in der Glutamin / Glutamat-fraktion in mikrobiell fermentierten Teigen wesentlich höher. *Lactobacillus sanfranciscensis* und *L.*

reuteri setzten während Pufferfermentationen Glutamin in Glutamat um. Demnach sind Laktobazillen in der Lage *in situ* Glutamat zu bilden. Das verantwortliche Enzym wurde im Rahmen dieser Arbeit nicht charakterisiert. Glutamat wird in der Lebenmittelindustrie häufig als Geschmacksverstärker verwendet und in Konzentrationen von 0.2-0.5% zugesetzt. In Abhängigkeit des Hydrolysegrades, der Desamidierungsaktivität und der Sauerteigdosierung wird in hefegetriebenen Brotteigen eine Glutamatkonzentration von maximal 0.3% erreicht. Es kann daher erwartet werden, dass die Glutamatbildung von Laktobazillen einen wesentlichen Einfluss auf dem Brotgeschmack hat.

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9 Appendices

9.1 Descriptive analysis of *L. perolens* TMW1.501 sourdoughs and bread crumbs

Standard *L. perolens* TMW1.501-sourdough were compared to *L. perolens* TMW1.501-sourdoughs to which citrate was added at the start of the fermentation. Ten untrained panelists were asked to describe the difference between the aroma of the doughs. In a second test, bread crumbs were offered instead of preferments.

Table A. Descriptive analysis of *L. perolens* TMW1.501 bread crumbs

Probenart	Beschreibung[c]
Einzelprobe[a]	1. Säuerlich-käsig 2. irgendwelche Bakterien? 3. schweflich, säuerlich (essigsäure o.ä.) 4. Heuaroma, Weinaroma 5. riecht viel säuerlicher, ähnlich wie saure Milch, Yoghurt 6. frisch, gras, pfirsichfruchtyoghurt 7. säurereicher, nach yoghurt, Buttermilch 8. etwas säuerlicher als die anderen, der Hefegeruch kommt nicht so stark heraus, leichter Backpulvergeruch im Nachhinein 9. säuerlich 10. Buttermilch
Doppelprobe[b]	1. malzig-teigig 2. Hefen-geruch 3. mehlig, neutral 4. Hefe-aroma, teigaroma, ‚brotig' 5. riecht wie mehl mit Hefe 6. Mehl, malzig 7. eher süßlich, nach süßrahmbutter 8. eher typischer hefegeruch, -flacher, nicht so intensiv 9. mehlich 10. malz, wie bauernbrotkruste

[a]: Citrat-versetzte Probe
[b]: normales *L.perolens*-Sauerteigbrot
[c]: 1 bis 9: Antworte der jeweiligen Probanden

Table B. Descriptive analysis of *L. perolens* TMW1.501 bread crumbs

Probenart	Beschreibung[c]
Abweichende Probe[a]	1. Nussig (anfangs stark) 2. ‚heller / leichter' ; weniger intensiver geruch 3. säuerlich, leicht beißend 4. malzig-sauer 5. Buttermilch, yoghurt 6. leicht säuerlich und auch hefe-artig 7. nur schwacher Unterschied, staubig, etwas säuerlicher, weniger brotig als E / K 8. säuerlicher 9. ?
Doppelprobe[b]	1. Nussig leicht 2. leciht malzig; intensiver geruch, leicht kastanienartig 3. frisch neutral, nach Hefe 4. süßlich, brotartig 5. malz nuss 6. hefeartig 7. brot typischm angenehm, süß, leicht malzig 8. brotig-> nach frischem Brot 9. ?

[a]: Citrat-versetzte Probe
[b]: normales *L.perolens*-Sauerteigbrot
[c]: 1 bis 9: Antworte der jeweiligen Probanden

9.2 Gene sequence of *codY* in L. sanfranciscensis DSM20451^{T}

GTGGCTACATTACTTGAAAAAACACGTAAAATCACCGCGATTTTGCAAGATGGAGTGACC
GATTTGCAACAAGAGTTGCCATACAACAGTATGACTGAACGCTTGGCAAACGTCATTGAT
TGCAACGCCTGCGTGATTAATACGAAGGGCGAGTTGCTTGGTTACTCATTGCCTTACAAT
ACAAACAATGATCGCGTTGACCAATTTTTCTACGATCGTAAATTGCCTGACGAATACGTT
CGTGCAGCAGTACGTATTTACGATACAATGGCAAACGTTCCTGTTGATCGTCCTTTAGCA
ATTTTCCCAGAAGAAAGTCTTAGCGATTTTCCAAAAGGTGTAACAACTTTAGCGCCTATCT
ATGGTTCTGGAATGCGTCTTGGAACATTTATTATGTGGCGTGAAGATGGTGAATTTACAG
ATGACGATCTTGTTTTGGTTGAGCTTGCAACAACAGTAATCGGTGTACAACTCTCAAACCT
TAAACTTGAACAAATGGAAGAAAATATCCGTAAAGACACTATGGCAACAATGGCTGTTA
ATACACTTTCTTACTCAGAAATGAAAGCTGTCAAAGCAATTATTGAAGAACTTGATGGTG
AAGAAGGGCATGTTATTGCCTCTGTCATTGCTGACAAGATTGGTATTACACGTTCAGTGA
TTGTTAATGCTTTACGTAAACTTGAATCTGCTGGTGTTATTGAATCACGTTCACTTGGTAT
GAAAGGAACTTATCTTAAAGTTCTTAATACTGGTTTGTTTGATAAACTTGCTGGACGTAAT
TTC

9.3 Gene sequences of the cytathionine lyase genes identified in this work

L. pontis TMW1.397
GTGTATGGGGGGACCTTCCGGCTGATCAACCAGGTCTTAAAGCGATTTGGCATGGAGTTC
ATGGTCGTCGACACCCGCGATCTGGCGGCTATCGAGGCGGCCATCCAGGATAACACCGTA
GCCATCTACTTTGAGACGCCGACCAACCCGCTCCTCCAGATTACCGACGTTCGGGCCGTG
ACCGTCCTGGCTCGCCGTCACCACCTTAAGACGATCGTTGATAACACCTTCGCGACTCCCT
ACAACCAGCGGCCGCTGACCCTGGGGGGCGGATGTCGTCGTTCACTCCGCCACTAAGTACC
TGGCTGGTCACAGTGACGTGGTGGCCGGGATTGCCGTCACCAACGACGCGGCGCTGGCG
GATCGGTTGGCCTTCCTCCAGAACTCCCTGGGAGCTACCCTGGGTCCAGACGACAGCTGG
CTGGTCCAACGGGGGATCAAGACCCTGGCGGCTCGAATGCGGATCCATGAGGAAAACGC
CGCCGCGGTGGTGGCCTTCCTCCAATCTGATGCGCACGTTGCCAAGATCTACTACCCGGT
CCTTCCGGACTTCCCAGGGCACGCGGTGGCCGCCAAGCAGACGCGCGGCTTTGGGGCGAT
GATTGCCTTTGAGCTCCGGGCTGGTCTGGATGTCAAGAAATTCGTCGAACACCTTCAATT
GATTGACCTTGCCGArwsCCTCGGCGGCAT

L. panis TMW1.648
GTGCACGGGGGGACATTCCGGTTAATCAATAAGGTATTAAAACGCTTTGGCCTTGAATTT
ACTGTTGTTGACATGCAAGACCTTGAAGCAGTCGAAAATGCAATTCAAGATAATACCGTT
GCAGTTTATTTTGAAACACCAACTAATCCGCTCTTGCAAATCGCTGATATTAAAGCAATTG
CTGATATCGCAAAAAAGCATGGAATAAAGACAATTGTTGATAATACCTTTGCTACTCCTT
ATAACCAACAACCGTTAACTCTTGGGGCAGATATTGTTGTTCACTCCGCAACCAAATATTT
AGGCGGCCATAGTGATGTTGTTGCCGGATTAGCAGTTACTAACGATGATGAAATTGCTGA
ACAATTAGCATTCCTGCAAAACTCAATCGGTAGTACGCTTGGTCCTGATGATAGTTGGCT
ACTACAACGAGGGATGAAAACTCTCGGTGCCCGGATGCGCGTTCACCAAGAAAATGCGA
ATGAAGTTATTAACTTCCTCCAAAATGATGACCATATTGGGAAAATTTATTATCCAGGCTT
AAAAGATTTTCCTGGTCATGAGGTTGCGGCTAAGCAAATGCGTAACTTTGGAGCAATGAT
CTCCTTTGAACTTAAGGATGGTTTAGATGCGAAGAAGTTTGTTGAAAGTCTACAATTGATT
GATCTTGCCGArwsCCTCGGCGGCAT

L. mindensis TMW1.1206
GTGCACGGGGGGACTTTCCGTTTAGTCAACAAAGTTCTCAAACGCTTTGGTTTAGAGTTC
AGTGTCGTTGATACCCGTGATCTCCAAACTGTTGAAGCTGCCATTCAACCAAATACCAAA
GCTATTTATTTTGAAACTCCTACTAATCCTTTACTAAAGATCACCGACATCGCTGCCGTCG
CCAAAATTGCGAAAGCTCACGATCTCAAAACAATTGTCGACAACACCTTTGCCACACCTT
ACAACCAAAATCCCCTTACACTTGGCGTTGATATCGTTGTTCACAGTGCCACTAAATACCT
CGGCGGACACAGTGATGTAGTAGCTGGTTTGGCCGTTACTAACGATGAAAAAATTGCTGA
AGACTTAGCCTTTTTACAAAATTCCATCGGAAGCGTCTTAGGTCCTGACGACAGTTGGCT
ATTGATGCGTGGTATTAAGACTCTAGGCGCCAGAATGCGTATCCACCACGAAAATGCCAA
ATTGGTTTACGATTATCTAGAAAATAATGACAAAGTTGCTAAAATTTACTATCCCGGTAA
TCCTGAATCAGTTGGCTACGACATCGCTAAAAAACAAATGCGTGGTTTTGGAGCCATGAT
CTCCTTTGAATTGAATGAAGGCTTGGATGCCAAAAAGTTTGTTGAAAGCTTACAATTGAT
TGACCTTGCTGArwsCCTCGGCGGCAT

L. reuteri TMW1.106
GATGTGTATGGGGGGACATTCCGGTTAATCAATAAGGTATTAAAACGATTTGGTCTTGAA
TTTACTGTTGTTGACATGCAAGACCTTGAAGCAGTAGAAAACGCAATTCAAGATAATACC
GTTGCAGTTTATTTTGAAACACCAACTAATCCGCTCTTACAAATCGCTGATATTAAAGCAA
TTGCTGACATCGCAAAGAAGCATGGAATAAAGACAATTGTTGATAATACCTTTGCTACTC
CTTATAACCAACAACCGTTAACTCTTGGGGCAGATATTGTTGTTCACTCCGCAACCAAAT
ATTTAGGCGGCCATAGTGATGTTGTTGCCGGATTAGCAGTTACTAACGATGATGAAATTG
CTGAACAATTAGCATTCCTGCAAAACTCAATCGGTAGTACGCTTGGTCCTGATGATAGTT
GGCTACTACAACGAGGGATGAAAACTCTCGGTGCCCGGATGCGCGTTCACCAAGAAAAT
GCGAATGAAGTTATTAACTTCCTCCAAAATGATGACCATATTGGGAAAATTTATTATCCG
GGCTTAAAAGATTTTCCTGGTCATGAGGTTGCAGCTAAGCAAATGCGTAACTTTGGAGCA

ATGATCTCCTTTGAACTTATGGATGGTTTAGATGCGAAGAAGTTTGTTGAAAGTCTACAAT
TGATTGATCTTGCCGAAwGCCTCGGCGGCATCGA

L. reuteri TMW1.976
GAAACGCCGACTACTCCGCTCTTACAAATCGCTGATATTAAAGCAATTGCCGATATCGCA
AAGAAGCATGGAGTAAAGACAATTGTTGATAATACCTTTGCCACTCCTTATAATCAACAA
CCATTAACTCTCGGGGCGGATATTGTTGTTCACTCCGCAACCAAATATTTAGGCGGTCATA
GTGATGTTGTTGCCGGATTAGCAGTTACTAACGATGATGAAATTGCTGAACAATTAGCAT
TCCTGCAAAACTCAATCGGTAGTACGCTTGGTCCTGACGATAGTTGGCTATTACAACGGG
GGATGAAAACTCTCGGTGCCCGGATGCGAGTTCACCAAGAAAATGCGAATGAAGTTATT
AACTTCCTCCAAAATGATGACCACATTGGAAAAATTTATTATCCAGGCTTAAAAGATTTC
CCTGGTCATGAGGTTGCAGCTAAGCAAATGCGGAACTTTGGAGCmATGATbkCCTTTG

L. fermentum TMW1.890
GTGCACGGGGGGACCTTCCGTTTGATCAACAAGGTCCTGAAGCGTTTCGGCCTCGAATTT
ACGGTTGTCGACACCCAGGACCTAGCGGCGGTCGAAGCGGCCGTGCAAGACAACACGGT
GGCCATTTACTTTGAAACACCCACCAACCCGCTCTTGGAGGTTTCAGACATCGAAGCGAT
TGCTAAGATCGCCAAGAAGCATGGCTTAAAGACGGTCATTGATAACACCTTTGCCACGCC
GTATAACCAACGCCCGCTGACCCTGGGCGCCGACATTGTAGTTCACTCTGCCACCAAGTA
CCTGGGCGGTCACTCCGACGTGGTGGGGGGGATTGCCGTTACGAACGACGAGGAAATTG
CCGAACAGTTGGCCTTCATCCAAAACTCGATTGGCGCCGTCCTTGGGCCCGACGACTCCT
GGCTTCTGATGCGGGGGATTAAGACCCTCGGCGCCCGAATGCGGATCCACCATGAAAAC
ACCGCCGCCGTCATTGAACTGTTGGAAAAGGACCCGCGGGTTGCTCGGGTACTGTACCCG
GGGCTGCCAGATTTCCCGGGACATGATATCGCGGCTAAGCAAATGGACCACTTCGGCGCC
ATGGTGTCCTTTGAACTGCAAACGGGGCTCTCGGCAAAGAAGTTTGTCGAAAGCCTGCAG
GTGATTACCCTAGCCGArwsCCTCGGCGGCAT

9.4 List of publications that resulted from this thesis

Original papers

Vermeulen, N., Pavlovic, M., Ehrmann, M. A.,Ganzle, M. G., R. F. Vogel, 2005, Functional characterization of the proteolytic system of Lactobacillus sanfranciscensis DSM 20451^{T} during growth in sourdough, Applied and Environmental Microbiology, Issue 71, 6260-6266

Vermeulen, N., Kretzer, J., Machalitza, H., Vogel, R. F., M. G Gänzle. Influence of redox-reactions catalysed by homo- and heterofermentative lactobacilli on gluten in wheat sourdoughs, Journal of Cereal Science, 2006, Issue 43, 137-143

Vermeulen, N., Gänzle, M. G., R. F. Vogel, Phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM20451^{T} and *Lactobacillus plantarum* TMW1.468, Journal of Agricultural and Food Chemistry, 2006, Issue 54, 3832-3839

Vermeulen, N., Czerny, M., Gänzle, M. G., Schieberle, P., R F. Vogel, Reduction of (E)-2-nonenal and (E;E)-2,4-decadienal during sourdough fermentation, Journal of Food Science, In press

Oral presentation

Nicoline Vermeulen, *Influence lactobacilli on redox-reactions*, FEMS, „8th Symposium on Lactic Acid Bacteria", August 2005

Poster presentations

Vermeulen, N., Thiele, C., Gänzle, M. G., R. F. Vogel, 2003, Cysteine metabolism by cereal-associated lactobacilli, In L. de Vuyst Sourdough from fundamentals to applications Brussels Vrije Uiversiteit Brussel (VUB) IMDO, 58-59,

Nicoline Vermeulen, *Influence lactobacilli on redox-reactions*, FEMS, „8th Symposium on Lactic Acid Bacteria", August 2005