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Contribution of thiol- and hydroxycinnamic acids metabolism of sourdough lactobacilli on structural and sensorial properties of wheat breads

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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 der Hilfe von Dritten zu Stande gekommen:

- Kapitel 2: Teigfermentationen (Referenzteige, organische Säuren, Glutathion) wurden teilweise von Dr. Nicoline Vermeulen durchgeführt. Sämtliche Daten wurden anhand von Zusatzmessungen bestätigt. Die Messungen des Kleberabbaus (2.2.10) wurden von Herrn Dr. Wieser vorgenommen. Die Brote wurden an der LfL (Landesanstalt für Landwirtschaft) durch erfahrene Bäcker hergestellt.
- Kapitel 3: Die Bestimmung der freien Ferulasäure wurde durch Herrn Dr. Wieser vorgenommen. Teilweise wurden Daten (Abbildungen) dieses Kapitels aus Diplom / Semesterarbeiten von Michael Novakowski und Wolfgang Holzmüller übernommen. Das Screening auf Decarboxylasen (3.2.6) wurde parallel auch von Dr. Susanne Kaditzky / Kristina Gramlich bestätigt. Die Untersuchung an Würzefermentationen und MRS-Medium mit zugesetzter Ferulasäure bzw. Coumarsäure wurde von Dr. Susanne Kaditzky vorgenommen. Die Bestimmungen von flüchtigen Aromakomponenten mittels GC/MS wurden am Hans-Dieter-Belitz Institute for Cereal Grain Research (hdbi) durchgeführt.
- Kapitel 4: Die Erzeugung der *nox*-Mutante wurde von Dr. Simone Freiding während ihrer Diplomarbeit durchgeführt. Genetische Daten von *Lactobacillus sanfranciscensis* TMW1.1304 wurden aus der veröffentlichten Arbeit von Prof. Dr. Vogel entnommen. Die intrezellulären Mangangehalte (4.2.9) in *Lactobacillus sanfranciscensis* wurden von Herrn Dr. Jürgen Behr bestimmt.

André Jänsch, Wädenswil

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PREFACE

This study was carried out at the department of “Technische Mikrobiologie Weihenstephan” (TMW) at the Technical University of Munich (TUM), and included a scientific visit at the Department of Agricultural, Food and Nutritional Science of the University of Alberta, Edmonton (Canada) during the years 2004 – 2008. Part of the research was funded by the Research Association of the German Food Industry (FEI) and the German Federation of Industrial Research Associations “Otto von Guericke” project No. AiF-FV 14492 N “Optimization of gluten quality in wheat doughs containing sourdoughs”, and the Canada Research Chairs Program. The participating sourdough company Ernst Böcker GmbH & Co. KG is gratefully acknowledged for funding part of my salary.

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I would like to thank my parents for putting me on this planet and to grow up without a care in the world. Finally, I am deeply grateful to Melanie – as of late Magdalena and Ludwig – for bringing so much joy, love and inspiration to my life.

SUMMARY

The contribution of wheat sourdough fermentations on the final bread volume is presented in the first part of this thesis. Exogenous and endogenous factors, e.g. strain selection, sourdough dosage, and fermentation period, which have a main impact on gluten quality were evaluated in respect to the final bread volume during wheat sourdough fermentations. A distinct classification was not observable for different microorganisms in sourdough fermentations with respect to the final bread volume. Moreover, the influence of acid supplementation resulting in different bread dough pH-values on bread volume is presented. Increasing amounts of acetic acid resulted in collapsed final bread volumes; thus, the performance of the baker´s yeast was strongly inhibited with elevated acetic acid concentrations. Redox reactions catalyzed by the fermentation flora during wheat sourdough fermentations generated reduced thiol compounds. Low-molecular weight compounds are capable bread aroma precursors; in addition, the dough rheology is affected by degradation of gluten proteins caused by a decrease of the redox potential in the dough system. The addition of baking improvers in fermentations with *L. sanfranciscensis* had a positive effect on the final bread volume; thus, the baking agents were not diminished during wheat sourdough fermentations.

The distribution of enzyme activities in lactobacilli able to generate volatile aroma compounds from hydroxycinnamic acids is discussed in the second part of this thesis. Ferulic acid was accumulated in wheat sourdough fermentations with *L. sanfranciscensis*; by using plate screening approaches the ability of lactobacilli was evaluated to release bonded hydroxycinnamic acids. Furthermore, predicted cinnamoyl esterases, and arabinoxylan-degrading enzymes were heterologously expressed and characterized of different lactobacilli. It was shown that liberated phenolic compounds could be further metabolized and account for aroma in sourdough fermentations. The free form of the phenolic acid is the substrate of decarboxylases by lactobacilli, generating volatile precursors, e.g. 4-vinylphenol and 4-vinylguaiacol, respectively. In wort fermentations with a combination of aroma active starter cultures, the results from the screenings were merged together; a combination of *L. brevis* TMW1.1326 and *L. pontis* TMW1.1086 showed capable results related to the generation of active aroma odors.

Thirdly, insights are given into the oxidative stress response of *L. sanfranciscensis*; two main enzyme activities contributing to the aerobic life of the strain, glutathione reductase and NADH-oxidase, respectively, are introduced, and characterized by creating of cognate mutant

strains. The loss of GshR activity in *L. sanfranciscensis* TMWΔ*gshR* resulted in a loss of oxygen tolerance. The *gshR*-mutant strain exhibited a strongly decreased aerobic growth rate on mMRS when compared to either anaerobic growth or the wild type strain; aerobic growth was restored by addition of cysteine. In addition, by inactivation of the native NADH-oxidase gene, it was ensured that besides fructose O_2 can react as an electron acceptor. The mutant strain was only able to grow in MRS media supplemented with fructose in aerated cultures, whereas the wild type strain showed a fructose-independent growth response. Additionally, *L. sanfranciscensis* showed manganese-dependent growth response in aerated cultures, the final OD and growth velocity was increased in media supplemented with manganese. Finally, the mutant strain TMW1.53Δ*nox* was more sensitive to the superoxide generating agent paraquat and showed inhibition of growth on diamide-treated MRS-plates without fructose supplementation. A scheme is proposed for the enzymes included in the detoxification of reactive oxygen species, and possible effects are shown of antioxidative compounds in *L. sanfranciscensis* to maintain redox homeostasis during the aerobic life of the strain.

ZUSAMMENFASSUNG

Der erste Teil dieser Arbeit präsentiert den Beitrag von fermentierten Weizensauerteigen auf das resultierende Brotvolumen. Exogene und endogene Faktoren, wie Stammauswahl, Sauerteiganteil und Fermentationsdauer, die einen grossen Einfluss auf die Kleberqualität und somit das resultierende Brotvolumen haben wurden während einer Weizensauerteigfermentation bewertet. Eine eindeutige Klassifizierung auf das resultierende Brotvolumen von verschiedenen, in Sauerteigen eingesetzten Mikroorganismen konnte nicht aufgestellt werden. Darüber hinaus ist der Einfluss von Säurezugabe, resultierend zu unterschiedlichen Brotteig pH-Werten, auf das finale Brotvolumen dargestellt. Steigende Mengen an zugesetzter Essigsäure lassen die Brotvolumen kollabieren. Folglich wurde die Leistung der Backhefe mit steigenden Essigsäurekonzentrationen stark gehemmt. Während einer Weizensauerteigfermentation werden Redoxreaktionen durch die Fermentationsflora katalysiert, die reduzierte Thiolkomponenten erzeugen. Verbindungen mit niedrigem Molekulargewicht sind mögliche Brotaromenvorstufen. Des Weiteren wird die Teigrheologie durch den Abbau des Klebernetzwerkes beeinflusst, welcher durch die Erniedrigung des Redoxpotentials im Teigsystem hervorgerufen wird. Der Zusatz von Backmitteln in Fermentationen mit *L. sanfranciscensis* hatte einen positiven Einfluss auf das resultierende Brotvolumen. Dementsprechend wurden die Backmittel während einer Weizensauerteigfermentation in ihrer Wirkung nicht abgeschwächt.

Der zweite Teil dieser Arbeit widmet sich der Verbreitung von Enzymaktivitäten in Laktobazillen, welche flüchtige Aromakomponenten aus Hydroxy-Zimtsäuren generieren. Ferulasäure wurde während einer Weizensauerteigfermentation mit *L. sanfranciscensis* angehäuft. Die Fähigkeit von Laktobazillen gebundene Hydroxy-Zimtsäuren freizusetzen, wurde mittels Platten-Untersuchung evaluiert. Weiterhin wurden mögliche Zimtsäureesterasen und Arabinoxylan-abbauende Enzyme von verschiedenen Laktobazillen heterolog exprimiert und charakterisiert. Es wurde gezeigt, dass freigesetzte Phenolverbindungen verstoffwechselt wurden und somit zur Entwicklung des Aromas in Sauerteigfermentationen beitragen können. Die freie Form der Phenolsäure dient als Substrat für Decarboxylasen von Laktobazillen, die flüchtige Verbindungen wie 4-Vinylphenol bzw. 4-Vinylguaiacol erzeugen. Die Resultate der Screenings wurden in Vorderwürzefermentationen mit einer Kombination von aroma-aktiven Starterkulturen zusammengefügt. Viel versprechende Resultate im

Hinblick auf die Erzeugung von aktiven Aromenverbindungen konnten mit einer Kombination von *L. brevis* TMW1.1326 und *L. pontis* TMW1.1086 erzielt werden.

Im dritten Teil der Arbeit ist ein Einblick in die oxidative Stressantwort von *L. sanfranciscensis* gegeben. Zwei wichtige Enzymaktivitäten (Glutathion-Reduktase und NADH-Oxidase) werden vorgestellt, die zum aeroben Leben des Stammes beitragen. Die beiden Enzymaktivitäten werden durch die Erzeugung von verwandten Mutantenstämmen charakterisiert. Der Verlust der Glutathion-Reduktase Aktivität in *L. sanfranciscensis* TMWΔ*gshR* ist verantwortlich für den Verlust der Sauerstofftoleranz des Stammes. Im Verhältnis zu anaeroben Wachstumsbedingungen bzw. dem Wachstum des Wildstammes zeigte der *gshR*-Mutantenstamm eine stark verringerte Wachstumsrate unter aeroben Bedingungen. Durch den Zusatz von Cystein zum Nährmedium wurde das Wachstum unter aeroben Bedingungen wiederhergestellt. Durch die Inaktivierung des ursprünglichen NADH-Oxidase Genes wurde zusätzlich sichergestellt, dass neben Fruktose auch Sauerstoff als Elektronenakzeptor verwendet werden kann. Der Mutantenstamm war nur in MRS-Medium mit zugesetzter Fruktose befähigt unter aeroben Kulturbedingungen zu wachsen, wohingegen der Wildstamm ein fruktose-unabhängiges Wachstumsverhalten aufwies. Des Weiteren zeigte *L. sanfranciscensis* unter aeroben Kulturbedingungen ein mangan-abhängiges Wachstums-verhalten. In Medium mit zugesetztem Mangan waren die optische Dichte und die Wachstumsgeschwindigkeit gesteigert. Abschliessend zeigte der Mutantenstamm TMW1.53Δ*nox* ein sensitives Verhalten gegenüber Paraquat, eine Substanz die Superoxide erzeugt. Auf MRS-Platten ohne Fruktosezusatz konnte ebenfalls eine Wachstumhemmung bei Diamid-Behandlung gezeigt werden. Eine Übersicht von beteiligten Enzymen die zu einer Überwindung von reaktiven Sauerstoffarten beitragen wurde aufgestellt, darüber hinaus werden mögliche Effekte von antioxidativen Verbindungen in *L. sanfranciscensis* gezeigt, welche einen Beitrag zur Redox-Selbstregulation während des aeroben Lebens des Stammes leisten.

1

1. GENERAL INTRODUCTION

This introduction reviews current knowledge of the topics exposed in this thesis. The main substrate wheat flour and the diversity of gluten proteins are described. A short introduction is given in the chemistry of gluten proteins, non-starch polysaccharides (NSP), as well as in the biodiversity and metabolic exploitation of wheat sourdough microbiota. *Lactobacillus sanfranciscensis* TMW1.53, the main strain used in the studies of chapter 2 and 4 is introduced. A general overview of oxidative stress responses is given for the *Lactobacillaceae.*

The second section focuses on wheat sourdough fermentations with lactobacilli. The evaluation of sourdough dosage and the impact on the final bread volume is shown; the effect of strain selection, fermentation period, organic acid accumulation, and addition of baking improver's is determined. Moreover, the influence of glutathione reductase activity of strain *Lactobacillus sanfranciscensis* TMW1.53 in sourdough fermentations is introduced, and the interaction of glutathione in respect to gluten quality is demonstrated.

Thirdly, the ability of hetero- and homofermentative lactobacilli is discussed to liberate phenolic aroma precursors in wheat sourdough fermentations. As a starting point for this study screening approaches with different lactobacilli were performed on mMRS plates supplemented with ethyl ferulate. Subsequently, with *in vivo* fermentations and molecular techniques the ability of specific *Lactobacillus* strains could be shown to have the potential of phenolic aroma enhancement during wheat sourdough fermentations. Main enzyme activities in the metabolism of bonded ferulic acid in wheat arabinoxylan to volatile phenolic aroma compounds of lactobacilli were heterologously expressed in *E. coli*.

Finally, the last chapter reviews the defenses against oxidative stress in *Lactobacillus sanfranciscensis* TMW1.53 on a genetically background. The detoxification of reactive oxygen species could be performed on an enzymatic level and on a non-enzymatic level as well. Two crucial enzyme activities were shut downed for cell defense against oxygen related stress by creating cognate mutants. An insight is summarized of the ability of *Lactobacillus*

sanfranciscensis to compensate the effect of reactive oxygen species during the aerobic life of the strain.

1.1 WHEAT FLOUR

Wheat is one of the three most important crops in the world, together with maize and rice (95). The vast majority of bread is traditionally produced from wheat flour. The gluten proteins, the non-starch polysaccharides, and lipids are the main compounds besides the major substance starch (42). During all steps of bread making, complex chemical, biochemical and physical transformations occur, which affect and are affected by the various flour constituents (42). The key characteristic of wheat is the unique properties of doughs formed from wheat flours; these properties depend on the structures and interactions of the grain storage proteins, which together form the "gluten" protein fraction (93).

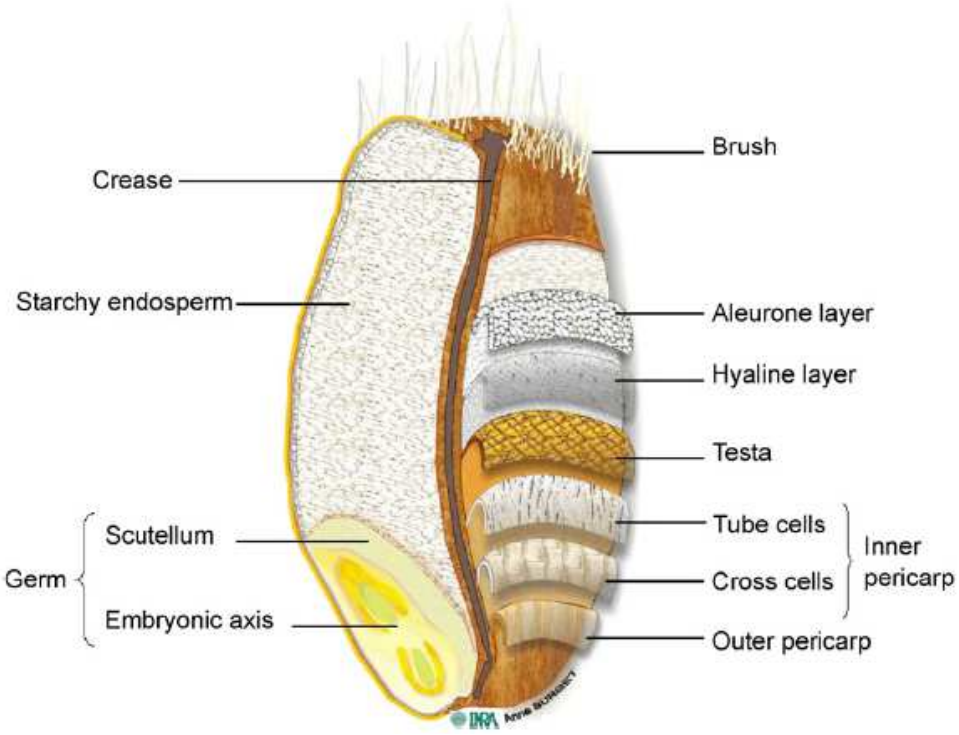

Figure 1. Wheat grain showing component tissues (Reprinted from Surget and Barron, 2005) (90).

The wheat grain contains of 2-3% germ, 13-17% bran, and 80-85% mealy endosperm (102). Outer layers of wheat grain (bran) have primarily a role of protection; cell walls in these tissues are thick, hydrophobic and essentially formed of cellulose and complex xylans, but also generally contain significant amounts of lignin (90). Proteins and carbohydrates each represent approx. 16% of total dry matter of the bran. The inner starchy endosperm mainly

contains carbohydrate reserves, which are needed for the growth of the seedling; apart from carbohydrates the endosperm contains lipids (1.5%), proteins (13%) and minerals (0.5-1.5%) (90, 102). The carbohydrate composition of wheat grain is important, because of the nutritional requirement of the sourdough microbiota in fermentations.

Table 1. Carbohydrate composition grain of wheat

Compound	**%**
Starch	63.0 – 72.0
Amylose (in starch)	23.4 – 27.6
Pentosan	6.6
β-Glucan	1.4
Fructosan	0.9 – 1.46
Glucose	0.02 – 0.03
Fructose	0.02 – 0.04
Sucrose	0.57 – 0.80
Maltose	Traces
Raffinose	0.2 – 0.39

(12)

Wheat flour mainly consists of starch (70–75%), water (approx. 14%) and proteins (10–12%). In addition, non-starch polysaccharides (2–3%), in particular arabinoxylans (AX), and lipids (1.5–2.5%) are important minor flour constituents relevant for bread production and quality (42). The starch is the most abundant component of wheat flour; it consists of branched glucose-units linked together by glycosidic bonds. Starch is composed of two different molecules:

- linear and helical amylose (500 – 6.000 α-(1-4) linked D-glucose-units).
- branched amylopectin (300.000 – 3.000.000 D-glucose-units linked together with α-(1-4) and α-(1-6) bonds).

Starch could be found in wheat in two different granules; the large, lenticular (B-type) granules having diameters from 10–35 nm, and the small, spherical (A-type) granules with a diameter of 2–10 nm (50). The amylose/amylopectin ratio differs between starches, but typical levels of amylose and amylopectin are 25–28% and 72–75%, respectively (24).

1.2 WHEAT GLUTEN PROTEINS

Gluten proteins play a key role in determining the unique baking quality of wheat by conferring water absorption capacity, cohesivity, viscosity and elasticity on dough (119). Scientific study of cereal grain proteins extends back for over 250 years, with the isolation of wheat gluten first being described in 1745 from Beccari, and more systematic studies have been carried out by Osborne (1859-1929) (94). Osborne (1924) classified the proteins of wheat according to their solubility in four different fractions: albumins, soluble in water; globulins, soluble in salt solutions; gliadins, soluble in 70% ethanol; glutenins, soluble in diluted acetic acid. Albumins and globulins represent about 20% of total proteins in wheat flour, gliadins and glutenins account for approx. 80% of the wheat flour proteins (12, 91, 93). This definition has since been extended to include related proteins, which are not soluble in alcohol-water mixtures in the native state. In wheat, these groups of monomeric and polymeric prolamins are known as gliadins and glutenins, respectively, and together form gluten proteins (1, 98, 119). Both fractions consist of numerous, partially closely related protein components characterized by high glutamine and proline contents (94, 119). Wheat prolamins are the major storage proteins present in the starchy endosperm cells of the grain (1).

Table 2. Classification of different wheat proteins

Osborne Fraction	**Solubility behavior**	**Composition**	**Functional role**
Albumin	water	Non-gluten proteins mainly monomeric	Variable
Globulin	dilute salt	Non-gluten proteins mainly monomeric	Variable
Gliadin	aqueous alcohol	Gluten proteins (mainly monomeric gliadins and LMW glutenins)	Dough viscosity
Glutenin	dilute acetic acid	Gluten proteins (mainly HMW glutenin polymers)	Dough elasticity
Residue	unextractable	Gluten proteins (HMW polymers and polymeric non-gluten proteins (triticins)	Variable

(42)

1.2.1 Gliadins and glutenins

Gluten proteins enable the formation of a cohesive visco-elastic dough that is capable of holding gas produced during fermentation and oven-rise, resulting in the typical fixed open foam structure of bread after baking (114). Both fractions are important contributors to the rheological properties of dough; nonetheless, their function related to dough quality are diverse. Gliadins and glutenins are usually found in more or less equal amounts in wheat (42). Hydrated gliadins have little elasticity and are less cohesive than glutenins; they contribute mainly to the viscosity and extensibility of the dough system. In contrast, due to their large size, glutenin polymers form a continuous network that provides strength and elasticity to the dough (42, 119). For example, highly elastic doughs are required for bread making, whereas more extensible doughs are needed for making cakes and biscuits (94, 95).

Gliadins are mainly monomeric proteins with molecular weights (Mw) around 28.000–55.000 and can be classified according to their different primary structures into α/β-, γ- and ω-type gliadins (114, 119). Cysteine residues play an important role in the structure of both gliadins and glutenins. These cysteine residues are either involved in disulfide bonds within the same polypeptide (intra-chain disulfide bonds) or in disulfide bonds between different polypeptides (inter-chain disulfide bonds) (114). α/β-Gliadins contain six cysteine residues and form three intra-chain disulfide bonds, while γ-gliadins contain eight cysteine residues and form four intra-chain disulfide bonds, ω-gliadins lacking cysteine residues and therefore the possibility to form disulfide cross links. Almost all cysteine residues are located in the C-terminal domain (43, 108). Minor portion of gliadins have an odd number of cysteines and are linked together or to glutenins; these gliadins are proposed to act as a terminator of glutenin polymerization. ω-Gliadins are characterized by the highest contents of glutamine, proline and phenylalanine which together account for around 80% of the total composition (119). Studies on the secondary structure have indicated that the N-terminal domains of α/β- and γ-gliadins are characterized by β-turn conformation, similar to ω-gliadins (64, 119). The non-repetitive C-terminal domain contains considerable proportions of α-helix and β-sheet structures. Comparison of amino acid sequences revealed that α/β-gliadins and γ-gliadins are both related to the LMW-GS, they are accordingly classified as “sulfur-rich prolamins” (98, 114).

The glutenin fraction has been difficult to study since it comprises several dozen different disulfide-bonded polypeptides in the molecular mass range of millions to hundreds of millions (64). The largest polymers termed “glutenin macropolymer” (GMP) showed the greatest contribution to dough properties; the amount in wheat flour (~ 20–40 mg/g) is strongly

correlated with dough strength and loaf volume (119). After reduction of disulfide bonds, the resulting glutenin subunits show solubility in aqueous alcohols similar to gliadins (119). Shewry and Halford classified all prolamins of the *Triticeae* (wheat, barley and rye) to three broad groups: sulfur-rich (S-rich), sulfur-poor (S-poor) and high molecular weight (HMW) prolamins (94). Based on primary structure, glutenin subunits have been divided into high-molecular-weight (HMW) subunits (MW: 67.000–88.000) and low-molecular-weight (LMW) subunits (MW: 32.000–35.000) by Wieser (2006) (119). Although LMW subunits show strong similarities with α-type and γ-type gliadins, they differ in one very important characteristic; apart from intra-chain disulfide bonds, inter-chain disulfide bonds that lead to incorporation of LMW subunits in glutenin polymers also occur in LMW subunits (114). LMW subunits contain eight cysteins, six of them are in positions homologous to α/β- and γ-gliadins, and therefore are proposed to be linked by intra-chain disulfide bonds (43, 119, 120). The two additional cysteine residues are not able to form intra-chain bonds; thus, inter-chain disulfide bonds are generated with cysteines of other gluten proteins.

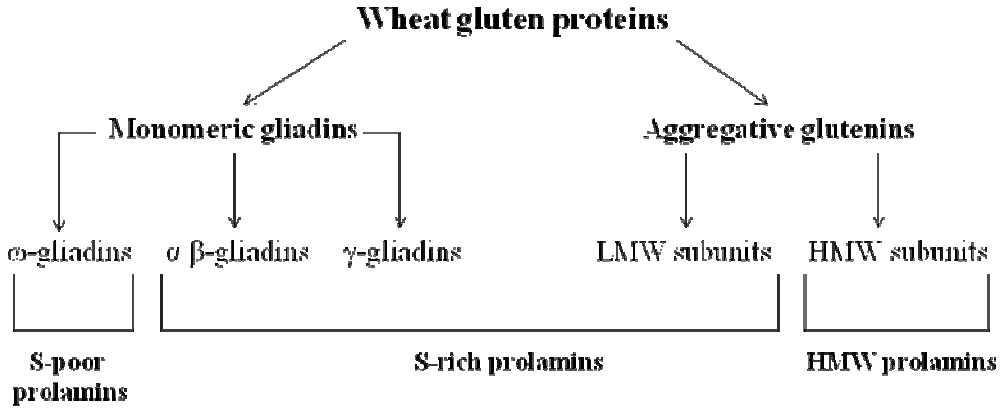

Figure 2. Classification of gliadin and glutenin subunits in wheat flour (64).

The current nomenclature of HMW subunits contains, besides an indication of the genetic locus (1A, 1B, or 1D) and the type (*x* or *y*), a number that was originally meant to reflect a ranking according to decreasing molecular weight; this number reflects now the mobility of the glutenin subunit on SDS-PAGE (114). HMW glutenin subunits belong to the minor components within the gluten protein family (~ 10%). Each wheat variety contains three to five HMW subunits which can be grouped into two different types, the *x*- (MW: 83.000–88.000) and the *y*-type (MW: 67.000–74.000), respectively (75, 119). HMW subunits have high contents of proline, glutamine, glycine, and low contents of lysine; they consist of non-repetitive N- and C-terminal domains (A- and C domain) flanking a central repetitive domain

(B domain) that confers elasticity to protein molecules (1, 40, 119). Domains A and C are characterized by the frequent occurrence of charged residues and by the presence of most or all cysteine residues (119).

Table 3. Repeating motifs of *x*- and *y*-type HMW subunits

	Subunits	
Motif	***x*-type**	***y*-type**
Tri-peptide	GQQ	--
Hexa-peptide	PGQGQQ	PGQGQQ
Nona-peptide	GYYPTSPQQ	GYYPTSLQQ

(96, 114)

The most important difference between the *x*- and the *y*-type lies within the A- and B domains and the occurrence of cysteine residues. The *x*-type except subunit Dx5 has four cysteines, three in domain A and one in domain C (97, 119). Two residues of domain A are linked by an intra-chain bond, the other two by inter-chain disulfide bonds. Subunit Dx5 has an additional cysteine residue at the beginning of domain B; it is proposed that another inter-chain bond is generated. The *y*-type has five cysteines in domain A and one in each of domains B and C. Inter-chain disulfide bonds have been detected in the cysteines of domain A, which are connected with corresponding residues of another *y*-type. Cysteine residue of domain B is linked together with cysteines of LMW subunits; at present, no inter-chain bond is observed of the cysteine residue of domain C (59, 119). The amino acid composition of HMW subunits indicates the hydrophilic nature of the central repetitive domain and the hydrophobic characteristics of the N- and C-terminal domains (96).

HMW subunits are the major determinants of dough and gluten elasticity; the two main features of the HMW subunits structure in respect to their role in glutenin elastomers are as follows: the number and distribution of disulfide bonds and the properties and interactions of the repetitive domains. Although disulfide-linked glutenin chains provide an “elastic backbone” to gluten for interactions with other glutenin subunits and with gliadins, evidence from spectroscopic studies of HMW subunits suggests that non-covalent hydrogen bonding between glutenin subunits and polymers may also be important (1, 7, 8, 117). The importance of non-covalent bonds can be demonstrated by the effects of agents such as salts, urea, and deuterium oxide on dough mixing (27, 112). Moreover, it was proposed by Belton that inter-chain hydrogen bonds formed in particular between glutamine residues are also important in

conferring elasticity (6, 94). The disulfide structure of native glutenins is not in a stable state, but undergoes a continuous change from the maturing grain to the final end product bread (120, 121). Differences in the disulfide bonding properties of glutenin subunit impact on their association within the glutenin macropolymer and role in establishing gluten structure and subsequent function (64). Different factors, e.g. genetic configuration (ratio: gliadin, LMW subunits, and HMW subunits), environmental differences (sulfur deficiency, heat or water stress), and the redox state of the system (presence of reducing or oxidizing agents) are influencing the state of disulfide structure (121). Hence, oxidizing and reducing agents which have a strong impact on the dough thiol-disulfide system can affect the polymerization of glutenin subunits and thereby change the rheological properties of the dough (36, 42). Already very low concentrations of endogenous glutathione drastically weaken the dough and increase extensibility through thiol/disulfide interchange. Reduced glutathione (γ-GluCysGly, GSH) and oxidized glutathione (GSSG) are both naturally occurring in wheat flour (61). These sulfhydryl compounds are capable of undergoing a disulfide-sulfhydryl interchange with other low-molecular weight thiol compounds as well as gluten proteins, resulting in the cleavage or reformation of disulfide bonds in wheat dough (43). The effect of sulfur (S) deficiency on the amount of total glutathione and cysteine in flour was evaluated by Köhler et al. (84); the different dough properties caused by S deficiency affected the final bread volume drastically. They concluded that the concentrations of glutathione and cysteine in S-deficient flours influenced the rheological dough properties at least as much as protein parameters.

Oxidizing or reducing agents that influence the thiol-exchange reactions between reduced glutathione and gluten proteins are therefore important components of baking improvers to standardize and to control dough rheology and bread texture in wheat baking processes. L-threo-ascorbic acid most strongly enhances strength, handling and baking properties of doughs; it is oxidized enzymatically by an endogenous ascorbic acid oxidase which uses molecular oxygen as an electron acceptor (43). Following, L-threo-dehydroascorbic acid and water are generated. The oxidized ascorbic acid acts as an electron acceptor in the oxidation of endogenous glutathione by wheat flour glutathione dehydrogenase. By this reaction the level of reduced glutathione is drastically decreased in the system, which can weaken the dough (36, 43). The influence of addition of exogenous oxidizing enzymes, e.g. tyrosinase, laccase, glucose oxidase, glutathione oxidase, and sulfhydryl oxidase could be seen in other studies. Also, the occurrence of dityrosine bonds during dough development is postulated to play only a minor role in the structure of wheat gluten (46). Nonetheless, the increase of

dehydro-ferulic acid-tyrosine (DFT) cross linking was detected during wheat dough mixing; therefore, it is very likely that DFT represents a new covalent cross link between AX and proteins in cereal flour (82). No correlation was observed between gluten yield and dityrosine concentrations, indicating that dityrosine cross links are not a determinant factor for gluten formation (87).

1.3 NON-STARCH POLYSACCHARIDES (NSP)

Beside starch and the gluten proteins, a third important group called non-starch polysaccharides (NSP) affects the process of bread production. As mentioned before, wheat flour consists to 2-3% of non-starch polysaccharides, in particular arabinoxylans (AX). In general, NSP includes arabinoxylan, β-glucan, cellulose, and smaller peptides derived from amylose and amylopectin (48). Nevertheless, these components have major effects on the use of wheat flour in bread making due to their viscosity in aqueous solution but also to their hydration properties (90). The wheat endosperm cell walls consist up to 75% (dry matter weight) of NSP, of which arabinoxylans are by far the most prominent group (85%) (70, 71). Arabinoxylans have a common structure of β-1,4-linked D-xylopyranosyl residues, substituted at the C(O)-3 and/or the C(O)-2 position with monomeric α-L-arabinofuranoside (78, 79). Some of the arabinose residues are ester linked on (O)-5 to ferulic acid (101). Polysaccharide-bond ferulic acid occurs widely in graminaceous plants, such as wheat bran and other cereals (32, 33). Hydroxycinnamic acids are very important compounds for the structure of the cell wall, because they can be coupled by peroxidase-mediated oxidative bonding to form a variety of diferulates enhancing the cross linking of polysaccharide chains (47, 83). On average 66% of xylosyl residues of the xylan backbone are unsubstituted. The arabinoxylans are grouped in water-extractable (WE-AX) and water-unextractable (WU-AX) AX (42). In wheat flours the average amounts of WE-AX and WU-AX are 0.5% and 1.7%, respectively (90). The content of ferulic acid in WU-AX is increased up to five-times in comparison with the content in WE-AX (81). The generation of diferulates via cross linking under oxidising conditions by WE-AX results in a strong viscosity increase of the arabinoxylan solution, and at high AX concentrations to a formation of a gel. Dehydro-diferulic acid cross-links are likely to be the major parameter that explains differences between WE-AX and WU-AX in the endosperm and aleuronic cell walls of cereal grains (2). Unlike most polysaccharide gels, arabinoxylan gelation process and gel properties are

governed by the establishment of covalent (e.g. diferulates) linkages and also weak hydrogen interactions. Moreover, the arabinoxylan properties are dependent on AX structural characteristics such as molecular weight (Mw), xylan backbone substitution (A/X ratio), as well as ferulic acid content and location (22, 113). The Mw of water extractable AX of wheat is in the range of 200-300 kDa (30). The arabinose to xylose ratio (A/X) is often used to characterise the structure of AX, and the average value of A/X ratio is 0.5 for WE-AX in the starchy endosperm (90). The structure of WU-AX is very close to that of WE-AX, but the average molecular weight and A/X ration are slightly higher for the water unexctractable AX compared to the water extractable AX (53). Ferulic acid is an important structural element of the AXs from starchy endosperm, although the amount linked to arabinoxylan is very low and represents 0.3% of WE-AX and 0.6-0.9% of WU-AX in wheat. These percentages correspond to about 2-4 ferulic acid residues per 1.000 xylose residues in WE-AX and 6-10 ferulic acid residues in WU-AX, respectively (10). The amount of dehydro-diferulic acids were detected 10-15 times in lesser amounts than ferulic acid in WE-AX, but only four times lesser amounts than ferulic acid in WU-AX (30, 62). In comparison, ferulic acid (FA) accounts for 0.9% of arabinoxylan in the pericarp from wheat, and very similar amounts are observed for dehydro-diferulic acids, which corresponds to about 30 ferulic acid residues per 1.000 xylose residues (2, 56, 69, 90). The presence of polysaccharide-bond hydroxycinnamic acids, e.g. *p*-coumaric, sinapic, and ferulic acid, in the cell walls of different plant species was observed in several studies (14, 19, 20, 23). They are found both covalently attached to the plant cell wall and as soluble forms in the cytoplasm (34). Ferulic and *p*-coumaric acid account for 0.66 and 0.004% dry weight of the cell wall in wheat bran, respectively (88, 101). Ferulic acid is linked to various carbohydrates as glycosidic conjugates, and it occurs as various esters and amides with a wide variety of natural products (88). A remarkable part of the ferulates in the cell wall form dimers, the presence of ester-linked dimeric phenolic compounds is a physiologically significant strategy to strengthen the cell wall, and to terminate cell wall extensibility. Ferulic acid also mediates polysaccharide-protein cross links via tyrosine or cysteine residues (65), as mentioned in chapter 1.2. FA exhibits a wide range of therapeutic effects against various diseases like cancer, diabetes, cardiovascular and neurodegenerative (4). It has been proved to be a potent antioxidant, reported to terminate free radical chain reaction and reduces the risk for coronary heart diseases (11). FA is an effective scavenger of free radicals and it has been approved in certain countries as food additive to prevent lipid peroxidation (103).

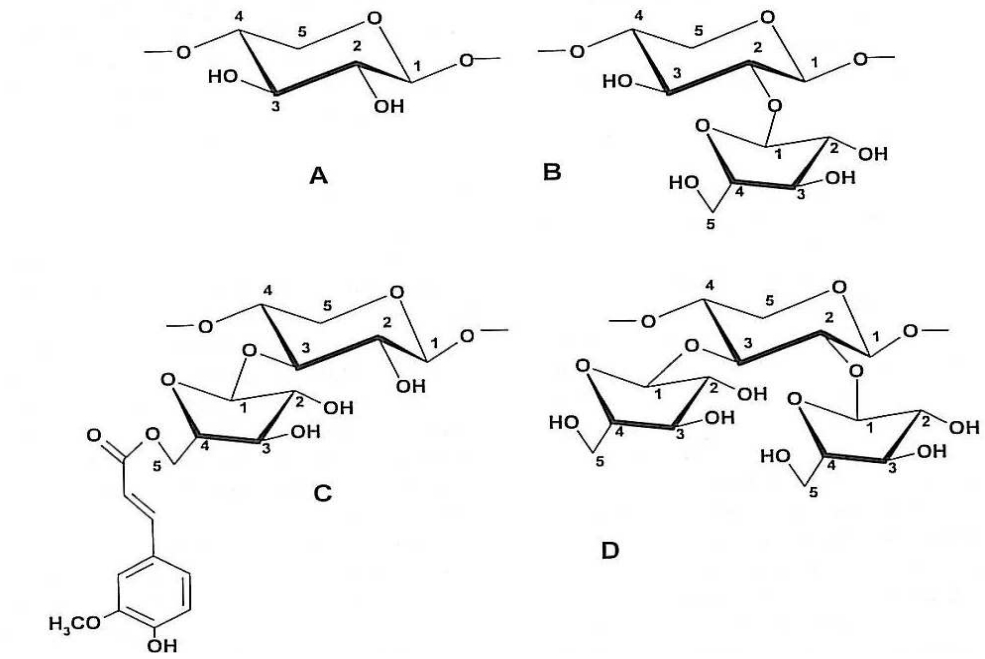

Figure 3. Structural elements of AX: (A) non-substituted D-xylopyranosyl residue; (B) D-xylopyranosyl residue substituted on C(O)-2 with a L-arabinofuranosyl residue; (C) D-xylopyranosyl residue substituted on C(O)-3 with a L-arabinofuranosyl residue and linkage of ferulic acid to C(O)-5 of the L-arabinofuranosyl; (D) D-xylopyranosyl residue substituted on C(O)-2 and C(O)-3 with L-arabinofuranosyl residue (42).

The amount and type of hydroxycinnamates vary greatly in respect to different cereal grains and different tissues, e.g. endosperm or bran, respectively.

WU-AXs are cross-linked in the cell wall structure and therefore water-unextractable. Until now, the optimal composition of arabinoxylans in respect to gas retention remains unclear, but the assumption that an increased WU-AX percentage led to decreased final bread volume is well accepted (26). Arabinoxylans are having a high water holding capacity; it has been proposed that in freshly prepared dough, AX hold up to one quarter of the water (42). Water soluble AXs act similar to gluten during wheat fermentations in respect to slow down the diffusion rate of carbon dioxide out of the dough, thus contributing to gas retention. Other studies postulated a positive influence of WE-AX to the formation of a secondary, weaker network enforcing the gluten network (51, 54). The effect of phenolic acids on the rheological properties and proteins of wheat dough and bread was evaluated by Han et al. (45); they observed that during bread making the wheat proteins were rearranged and the supplemented phenolic acid reduced high molecular weight proteins (45). In conclusion, phenolic acids affect bread making quality by altering the flour protein properties.

1.4 WHEAT SOURDOUGH MICROBIOTA AND LACTOBACILLUS SANFRANCISCENSIS

Sourdough is a mixture of wheat flour and water and is fermented with lactic acid bacteria (LAB) and yeasts over a certain period of time at indicated temperatures. The main function of wheat sourdough fermentations is leavening of the dough, producing of organic acids, and generating volatile aroma compounds, therefore improving the flavor and structure of the final bread (13). Some LAB are generating exopolysaccharides in the dough system, which have an impact on crumb structure and shelf life of sourdough breads. LAB growth in the sourdough may originate from selected natural contaminants in the flour or from starter cultures containing one or more known species (29). LAB are the predominant microorganisms and in many cases yeasts are present in significant numbers. Whereas in the majority of fermented foods homofermentative LAB play an important role, heterofermentative LAB are dominating in sourdough fermentations (29). The classification of different sourdough types could be seen elsewhere (9, 13, 29); nevertheless, the main strains used in this study *Lactobacillus (L.) sanfranciscensis* (main germ, type-I-sourdoughs), *L. brevis* (isolated from type-I-sourdoughs), and *L. pontis* (typical germ in type-II-sourdoughs) could be classified into the different types of sourdough based on their occurrence and optimal growth temperature. The strains are obligate heterofermentative microorganisms. The heterofermentative metabolism of hexoses was clearly pictured by Gänzle et al. (38, figure 4).

L. sanfranciscensis is the predominant key bacterium in traditionally fermented sourdoughs. The genomic analysis of *L. sanfranciscensis* by Vogel et al. (116) revealed a circular chromosomal sequence of 1.298.316 bp and two additional plasmids pLS1 and pLS2, with sizes of 58.739 bp and 18.715 bp, which are predicted to encode 1.437, 63 and 19 ORFs, respectively. The sequenced strain *L. sanfranciscensis* TMW1.1304 was isolated from a commercial mother sponge with a tradition of continuous propagation by back-slopping procedures (116). *L. sanfranciscensis* contains the smallest genome within the lactobacilli so far, but has the highest rRNA operon density (5.39 per Mbp). Thus, it was proposed that the high rRNA operon density allows *L. sanfranciscensis* to respond quickly to favorable growth conditions in the sourdough environment, finally resulting in high growth rates (116). Based on the genome sequence of *L. sanfranciscensis* TMW1.1304 it was proposed that the strain has the potential to synthesize *de novo* four amino acids (alanine, aspartate, glutamate, and glutamine). Moreover, among three other amino acids (arginine, lysine, asparagine) originaring from L-aspartate, L-alanine can be converted into L-cysteine.

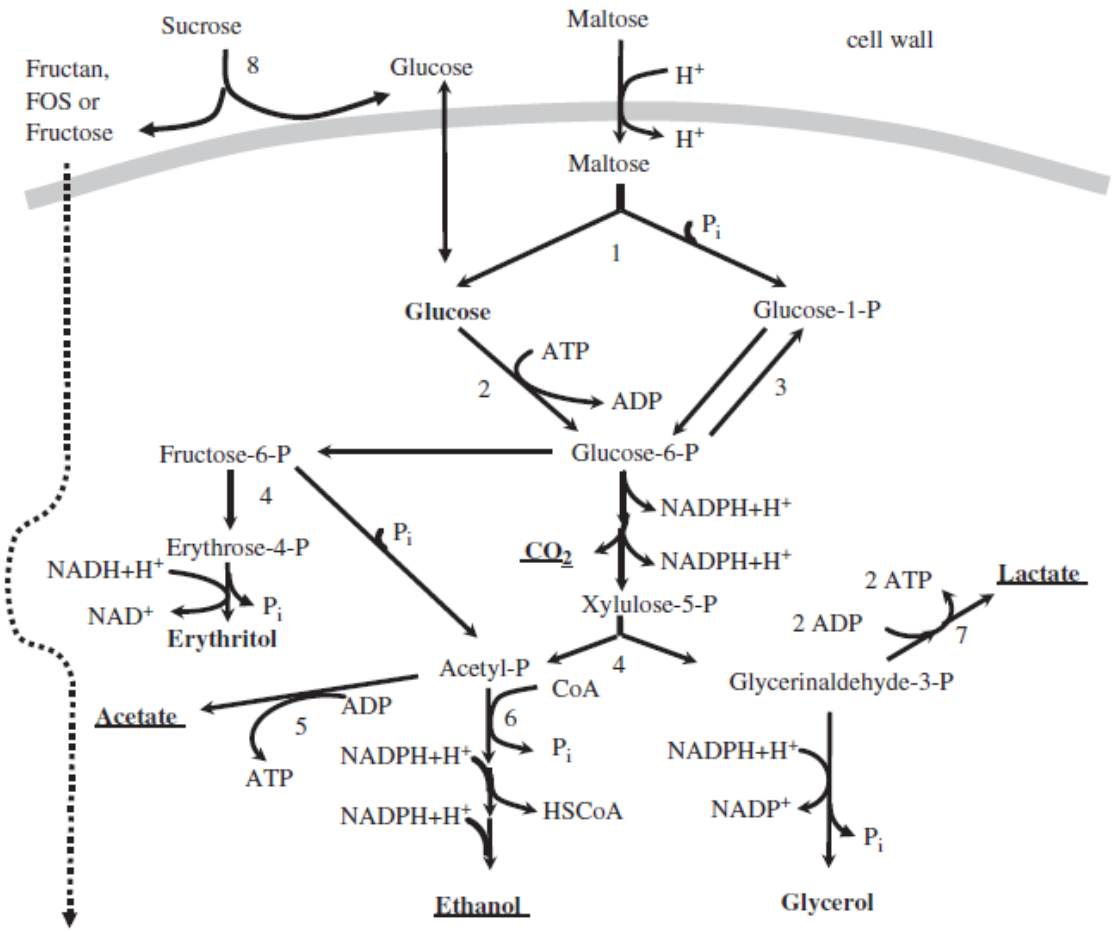

Figure 4. Maltose and sucrose metabolism of *L. sanfranciscensis*, updated from Hammes et al. (1996). End products of metabolism are printed in bold, major products of the metabolism in sourdoughs are underlined. Key metabolic enzymes of *L. sanfranciscensis* are indicated by numbers. 1. maltose-phosphorylase; 2. hexokinase; 3. phosphoglucomutase; 4. phosphoketolase; 5. acetate kinase; 6. phosphotransacetylase; 7. lactate dehydrogenase; 8. cell-wall bond fructosyltransferase (38).

The predicted auxotrophy for the remaining 12 amino acids could be explained with the presence of a large number of peptidases, proteases, and transport systems for amino acids and peptides (116). Besides several amino acid transporters with unknown specificity, ABC transporters for glutamine, methionine and cystine are predicted; the latter transporter for cystine in *L. sanfranciscensis* is also introduced in chapter 4 of this thesis. The regulatory system of *L. sanfranciscensis* is harbouring amongst others two genes for *rpoE*, a sigma factor involved in high temperature and oxidative stress response, and one gene for *rex*, a redox-sensing transcriptional repressor (116).

Sourdough fermentations are characterized by a highly stable association of yeasts and lactic acid bacteria. The synergistic interactions between lactobacilli and yeasts are based on the metabolism of carbohydrates and amino acid accumulation. Maltose is the preferred carbon

source for *L. sanfranciscensis* (38), and is available in high amounts in wheat sourdough fermentations due to the activity of endogenous amylases. Maltose-negative yeasts like *Saccharomyces exiguus* or *Candida humilis* could build a stable consortium with *L. sanfranciscensis* in wheat sourdoughs. *Lactobacillus brevis* and *Lactobacillus pontis* also exhibit maltose phosphorylase activity (106). Most of the obligatory heterofermentative lactobacilli metabolize pentoses, but not *L. sanfranciscensis* (44). A fructosyltransferase activity of *L. sanfranciscensis* TMW1.392 was shown by Tieking et al.; nonetheless, the majority of *L. sanfranciscensis* strains do not exhibit fructosyltransferase activity (111).

During the oxidation of fermentable substrates, reduced coenzymes in form of NADH or NADPH are generated; the transfer of electrons to external electron acceptors is maintaining the catabolic flux by regeneration of the oxidized form of the pyridine nucleotides (58). The utilization of electron acceptors in *Lactobacillus sanfranciscensis* was shown by Stolz et al. (105), fructose is used as an electron acceptor with the help of mannitol dehydrogenase generating mannitol. The utilization of electron acceptors enables the production of acetate from acetyl-phosphate and the synthesis of an additional ATP. Oxygen can also be used as an external electron acceptor by *L. sanfranciscensis* under aerobic conditions see chapter 4 and other studies (38, 58, 105). The growth of *L. reuteri*, *L. fermentum*, and *L. pontis* is inhibited under aerobic conditions due to the lack of NADH oxidase reactions (107). Several organic compounds, e.g. citrate, glutathione (GSSG), and long chain aldehydes can serve also as external electron acceptors for *L. sanfranciscensis* under static, anaerobic conditions (38).

1.5 OXIDATIVE STRESS RESPONSE IN LACTIC ACID BACTERIA

LAB have been found to contain unique flavoproteins that are different from the respiratory redox enzymes of cytochrome-containing bacteria like *Escherichia coli*. By maintenance of the redox balance of NAD/NADH under aerobic conditions, several enzymes are involved in the response to oxidative stress. While *L. sanfranciscensis* CB1 was described to produce a single Mn-containing superoxide dismutase (SOD) that is essential for aerobic growth, it lacks the respiratory chain and catalase enzyme (28). Other strains have not been investigated in this respect. Furthermore, lactic acid bacteria use NADH peroxidase activity to decompose H_2O_2 (25). NADH peroxidase resumes the role of heme-containing catalases by reducing intracellular levels of H_2O_2 while also regenerating oxidized pyridine nucleotide (89). Reduced coenzymes in form of NADH are accumulated during the oxidation of fermentable

substrates, to maintain the catabolic flux the oxidized form of this pyridine nucleotide must be regenerated. Under aerobic conditions two types of NADH oxidases, corresponding to H_2O_2-forming oxidase (Nox-1) and H_2O-forming oxidase (Nox-2) can contribute to this goal. In *Streptococcus mutans* Nox-2 was identified to play an important role in energy metabolism, while the role of Nox-1 was negligible in this respect (49). The FAD-dependent oxidoreductase, NADH oxidase (Nox-2), in *L. sanfranciscensis* catalyzes the direct four-electron reduction of O_2 to water and serves as an electron acceptor during active aerobic metabolism (86). The regeneration of two molecules of NAD^+ by Nox provides oxidized pyridine nucleotides for glycolysis. Furthermore, since Nox-activity directly reduces O_2 to H_2O without the formation of reactive O_2 intermediates, it may serve to protect *L. sanfranciscensis* against oxidative stress. It is suggested that peroxide is not released from the active site of Nox during regeneration of NAD^+ under aerobic conditions (86). NADH oxidases have been purified and characterized in *L. sanfranciscensis* (67, 68), in *Lactobacillus brevis* (52), and several other lactic acid bacteria, e.g. *Streptococcus pyogenes* (41) and *Lactococcus lactis* (73). Lactic acid bacteria show a greater metabolic potential when the reduced cofactors, from which NADH is the most significant, are regenerated by exogenous electron acceptors. In most cases, this also results in an increased fermentation yield. During growth of *L. sanfranciscensis* in a medium containing maltose, glucose and other electron acceptors like fructose an additional amount of ATP is generated by the metabolism of fructose. Aerobic growth of *L. sanfranciscensis* on maltose was found to result in a higher final cell yield and growth rate than anaerobic growth. The higher level of acetate production is concomitant with the production of ATP through the acetate kinase reaction (104). The shut-down of the ethanol branch of the phosphoketolase pathway in the presence of exogenous electron acceptors seems to be very common among heterofermentative lactobacilli.

Many lactic acid bacteria, especially lactobacilli, contain very high intracellular Mn(II) levels, e.g. *Lactobacillus plantarum* (3, 57) and *Lactobacillus brevis* (5). The manganese content acts as a scavenger of toxic oxygen species, or as a cofactor for several enzymes such as catalase (60) and SOD (37). Kehres and Maguire reviewed the Mn(II)-dependent enzymatic detoxification of oxygen radicals, and showed also the non-enzymatic influence of Mn(II) as a ‘free’ transition metal, substituting functions for iron and heme in a hitherto chemically undefined way (57).

Glutathione reductase (*gshR*) is a member of the family of flavoprotein disulfide oxido-reductases. The enzyme catalyzes the NADPH-dependent reduction of glutathione disulfide. In addition to the technological relevance in wheat doughs, glutathione has an important function as redox buffer in bacterial cells. Glutathione is the major non-protein thiol compound in living cells, it was found to be involved in the resistance to osmotic stress (99), toxic electrophiles (35) and oxidative stress (21, 100). Glutathione also acts as an electron donor for both scavenging of reactive oxygen, e.g. from respiration, and metabolic reactions such as reduction of hydroperoxides and lipidperoxides (72). The glutathione reductase plays an essential role in cell defence against oxygen stress by maintaining a high intracellular GSH/GSSG status (66). GshR has been purified and characterized from several bacteria, e.g. *Cyanobacterium anabaena* PCC7120 (55), *Pseudomonas aeruginosa* (80). In *Escherichia coli*, glutathione-based reduction systems contribute to the protection against oxidative stress (21); however, the glutathione reductase from *E. coli* seems to play a minor when compared to thioredoxin reductases (100). Lactic acid bacteria are known for their ability to accumulate reduced glutathione (118). A sulfhydryl uptake system was studied by Thomas (109) and the import and metabolism of glutathione by *Streptococcus mutans* was reported by Sherrill and Fahey (92). Glutathione reductases were characterized from *Streptococcus thermophilus* CNRZ368 (76) and *Enterococcus faecalis* (74) but their contribution to the oxygen tolerance of lactobacilli remains unknown. *Lactococcus lactis* exhibited higher levels of glutathione reductase activity when growing under aerobic conditions. The increased accumulation of GSH under aerobic conditions was interpreted as regulatory mechanism that protects the cells against oxidative stress (63). Vido et al. (115), showed that the thioredoxin reductase-thioredoxin system in *Lactococcus lactis* is important but not essential during aerobic life, suggesting the existence of an additional redox system that contributes to oxygen tolerance. Moreover, the respiration capacity of the fermenting bacterium *Lactococcus (Lc.) lactis* was confirmed in several studies (17, 31, 77). The damaging effects of oxygen on *Lc. lactis* cells were not observed when cells were grown in the presence of both oxygen and a heme source (31). In addition, aerated, heme grown *Lc. lactis* cells displayed increased growth yield, resistance to oxidative stress, and improved long-term survival when stored at low temperatures (17, 85). Heme is an essential cofactor of cytochrome complexes in the electron transport chains of respiring cells (39, 110). Duwat et al. (2001) provided genetic and additional biochemical evidence for respiration in *Lc. lactis*, the cydA (cytochrom *d* oxidase) gene is induced transcriptionally in late exponential phase of cells grown under respiration

conditions (31). The ability to utilize an external heme source indicated that a heme uptake mechanism must also be present in *Lc. lactis*; genes comprising the *ygfCBA* operon (ABC transporter) of *Lc. lactis* are involved in heme homeostasis (31, 77). The electron transport chains of *Lactobacillus plantarum* WCFS1 were evaluated under anaerobic respiration conditions; indicating that *L. plantarum* is able to reduce nitrate to nitrite only when both heme and menaquinone are supplied to the medium (15). Moreover, some LAB demonstrate respiratory behavior when menaquinone and/or heme are supplemented to the culture broth in a screening approach conducted by Brooijmans (18). However, very few LAB besides *L. plantarum,* e.g. *L. reuteri* and *L. fermentum* contain the necessary *narGHJI* genes for anaerobic nitrate respiration (15, 16).

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2

2. EVALUATION OF INFLUENCING FACTORS ON FINAL BREAD VOLUME OF SOURDOUGH PREPARED BREADS

The quality of baked goods from wheat attained with a straight dough process is determined predominantly by the quantity and quality of the gluten proteins of the flour. The importance of gluten proteins for the baking quality of wheat flours is explained by their ability to form high molecular weight aggregates that are stabilized by covalent bonds and non-covalent interactions between monomers (see also chapter 1). The use of sourdough in wheat baking improves the sensorial and nutritional quality of the products. Especially proteolytic events during fermentation are highly relevant for the aroma formation in wheat breads; however, the amount of the glutenin macropolymer is strongly reduced during sourdough fermentations (40). Because of the limited knowledge on the biochemical nature of gluten modifications during sourdough fermentations, it is currently not possible to optimize the gluten quality in wheat doughs with sourdough addition through a targeted selection of fermentation organisms and baking improvers. In addition to proteolytic events, the gluten quality is strongly affected by redox reactions (37). In contrast to proteolytic degradation, these reactions are reversible, and may be affected by appropriate baking improvers; therefore, appear advantageous as a tool to control gluten quality. Because sourdough fermentations are increasingly used in wheat baking, bakeries and suppliers require starter cultures and baking improvers that enable the optimization of gluten quality in wheat sourdoughs. Special attention is guided on the texture and volume of wheat bread produced with sourdough fermentation.

It was recently shown that *Lactobacillus sanfranciscensis* increases the levels of low molecular weight thiol components as well as thiol levels in gluten proteins during sourdough fermentation (37). The reduction of disulfide bonds by *L. sanfranciscensis* may determine gluten quality in addition to the pH-dependent activity of cereal proteases. The effect of *L. sanfranciscensis* on disulfide exchange reactions in wheat doughs was attributed to glutathione reductase activity of the microorganism (37). Glutathione reductase (GshR) is a member of the family of flavoprotein disulfide oxidoreductases. The enzyme catalyzes the NADH-dependent reduction of glutathione disulfide. The expression of the enzyme during

wheat sourdough fermentations with *L. sanfranciscensis* was shown, indicating that the glutathione reductase of *L. sanfranciscensis* is active in sourdough fermentations and thus may contribute to increased SH-levels in sourdough. The reduction of glutathione was evaluated in buffer-based fermentations with cells of *L. sanfranciscensis*, demonstrating the generation of GSH. A direct effect of *L. sanfranciscensis* was proposed unlikely on disulfide bonds in gluten proteins due to the inaccessibility of intracellular or cell-wall bond enzymes of the strain (37).

The aim of this part is to characterize modifications of gluten proteins in sourdough fermentations and their impact on bread quality. Major enzymatic reactions of flour and the fermentation flora are identified, which have an impact on the gluten network. Main focus is set on redox reactions catalyzed by glutathione dehydrogenases. A glutathione reductase knock-out mutant was constructed from *Lactobacillus sanfranciscensis* TMW1.53, and the impact during wheat sourdough fermentations on thiol levels was determined using the wild type strain and its cognate mutant. Moreover, the impact of the glutathione reductase is shown on gluten proteins (gliadins and glutenins, respectively) during wheat sourdough fermentations. The influence of sourdough supplementation on the baking performance of wheat flours is presented in respect to final bread volume. Knowledge about degradation and modification events of gluten proteins during wheat sourdough fermentations could lead to the development of new bakery improvers, which enhance bread quality based on rheological-technical parameters. The characterization of the reference doughs was performed using standard baking trials (Landesanstalt für Landwirtschaft LfL, Freising). The baked breads were classified according to their volume. Different dosages of sourdough (2%, 5%, 10%, and 20%) were added to directly produced bread dough. The breads and sourdoughs were made with the same flour. On the basis of experiments with different reference strains, all combinations were evaluated concerning strain selection and supplemented quantity of sourdough, which generated the maximum bread volume. In this study the application of ascorbic acid was not realized. Thus, the extensive influence of the substance on the redox system of gluten was eliminated, and possible differences caused from the used fermentation flora on bread volume could be seen as opposed to trials with flours supplemented with ascorbic acid.

2.1 MATERIALS AND METHODS

2.1.1 Used strains, medium, and cultivation conditions

Following main strains were used in this study: *Lactobacillus sanfranciscensis* TMW 1.53 (type-strain), *Lactobacillus plantarum* TMW 1.468, *Lactobacillus fermentum* TMW 1.890, *Lactobacillus sakei* TMW 1.22, *Lactobacillus reuteri* BR11, *Lactobacillus pontis* TMW1.84, and *Enterococcus faecalis* TMW 2.22. The strains were cultivated under anaerobic conditions at 30°C using modified MRS-medium (see chapter 5.1.7). The pH-value was adjusted to 6.2 before the sterilization step; sugars were autoclaved separately. For cloning approaches *E. coli* DH5α was used, and incubated aerobically in LB broth at 37°C. Genomic DNA's of different lactobacilli were used in the PCR screening approaches for glutathione reductase and cystathionine-γ-lyase genes in lactobacilli (see table 7).

2.1.2 Wheat and flour

For evaluation of the different degradation processes during wheat sourdough fermentations A, B and E-class wheat was used in this study. The species were selected after consultation with Bayerische Landesanstalt für Landwirtschaft (LfL), special attention was laid on frequently grown wheat species. Untreated A-class wheat grains cultivar "Tommy" was provided by Nordsaat Saatzucht GmbH (Boehnshausen, Germany) and milled by LfL. The grains were milled to flour type 550, and stored under cool and dry conditions until usage. The protein and water content of the flour was 13.4% and 14.1%, respectively. The selected species of B-class wheat cultivar "Dekan" and E-class wheat cultivar "Bussard" were provided by Lochow-Petkus GmbH (Bergen, Germany). All other wheat sourdough fermentations and bread doughs were prepared using commercially available flour without supplemented ascorbic acid (e.g. Meyermühle, Landshut). The determination of thiol levels in wheat sourdough fermentations with lactobacilli (see chapter 2.2.8 and 2.2.9) was performed with enriched wheat flour (ash content of 0.4-0.5 g/100 g) obtained at a local supermarket in Edmonton (Canada).

The federal office of plant varieties (Germany) classifies wheat species in four so-called baking quality groups. The main feature of classification is the final bread volume in baking trials with the Rapid-Mix-Test:

E-class: elite wheat; with excellent characteristics; mainly mixed with weaker wheat.

A-class: quality wheat; with high protein content; could compensate deficits of other species.

B-class: bread wheat; all species that are appropriate for production of pastries.
C-class: other wheat; used as foodstuff for both human and animal consumption.

2.1.3 Wheat sourdough fermentations

Cell cultures grown overnight were harvested, rinsed two-times with tap water and finally suspended in 25 ml tap water. Two doughs were made with the same starter culture; the culture suspension was mixed with 25 g of flour resulting in a sourdough with TA (dough yield) of 200. 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/g dough were added after 7 hours to adjust the pH to 3.6. Samples were drawn after 0, 5, and 24 (or 20) hours from the sourdough fermentations at 30°C. Glutathione (oxidized/reduced) was added with a concentration of 10 mM to dough. Total aerobic count was evaluated immediately; the other samples were stored at -20°C until usage.

2.1.4 Determination of cell counts and dough pH

In all doughs, the dough pH and the cell counts were determined as described by Thiele et al. (33). In chemically acidified doughs, cell counts remained generally $<10^4$ cfu/g. The absence of contaminants in sourdoughs was verified by observation of uniform colony morphology.

2.1.5 Dough characterisation and determination of bread volume using standard baking trial (Rapid-Mix-Test)

The identity of the fermentation flora of the used starter culture was evaluated using total aerobic count technique. The concentrations of organic acids, ethanol, and free amino nitrogen (FAN) were determined as performed by Thiele et al. (6, 33). For quantitative determination of free thiol-groups according to Antes and Wieser (2), the sourdoughs were extracted with a SDS-solution (1:10). 75 µl of the dough extract was mixed with 150 µl nitrogen-saturated reagent A (n-propanol (50%) in sodium phosphate buffer (50 mM, pH 8.0)) and 7.5 µl reagent B (39.6 mg Ellman's reagent (5,5'-dithiobis-(2-nitro benzoic acid))) in 10 ml sodium phosphate buffer (0.5 M, pH 7.0). The extracts were measured against a blank (sample approach without reagent) using a spectrophotometer at 412 nm. Different concentrations of glutathione served for set up of a calibration curve.
The influence of a sourdough dosage with selected starter cultures on the baking performance was evaluated in comparison to chemically acidified reference doughs using standard baking

trials related to Rapid-Mix-Test. The breads were characterized in reference to their volume. The Rapid-Mix-Test was developed at the Landesanstalt für Landwirtschaft (Freising, Germany) to evaluate the baking performance of flours; all parameters beside the flour were kept preferably constant. The reproducibility was high due to the fact that the baking process was performed by experienced bakers. As mentioned before, the addition of ascorbic acid was omitted.

Composition of the doughs: 1000 g flour, 1% peanut fat, 5% yeast, 1% sugar, 1.5% salt, 0.6 g malt, and 10% (or otherwise stated) sourdough were mixed with 570 ml water during one minute, resulted in homogenous dough. Subsequently, the dough rest was performed for 20 minutes at 32°C with a relative humidity of 80%. The dough pieces were formed and baked for 20 minutes after an additional dough rest of 30 minutes at 32°C and relative humidity of 80%. The evaluation of the breads volume using granulate measurement method was performed after a cool down phase of the baked breads.

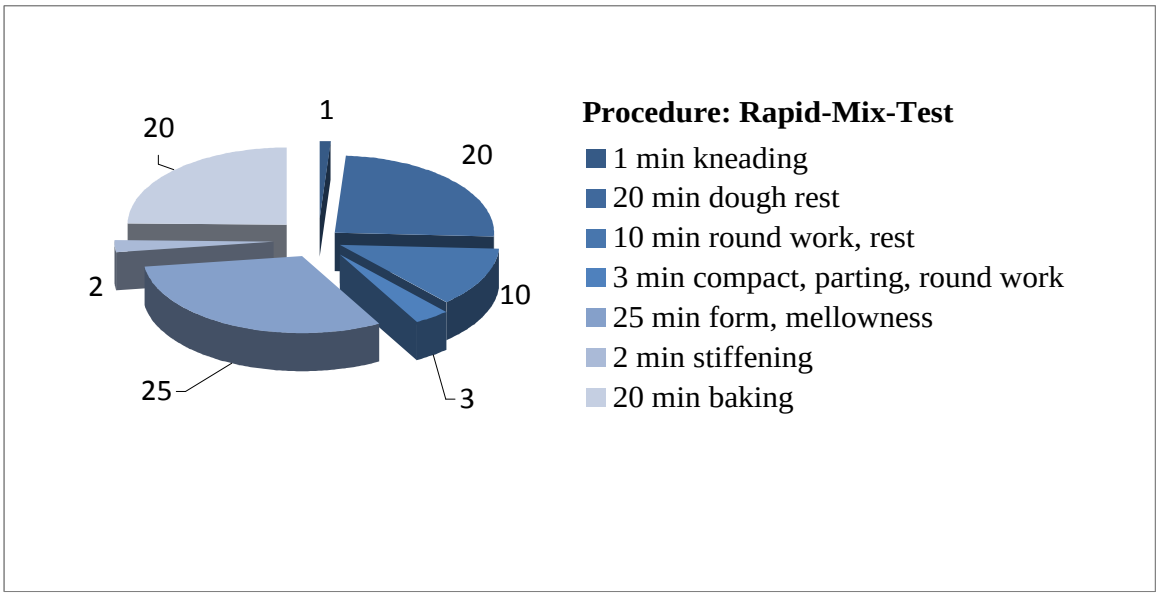

Figure 5. Procedure Rapid-Mix-Test developed at the LfL (Freising).

2.1.6 Determination of the final bread volume with a granulation method

The final volume of the baked breads was determined with a displacement method. Firstly, a container was filled with flaxseeds to evaluate the volume of the container. Following, the removal of the flaxseeds, and bread was placed into the container. Subsequently, the container was filled with flaxseeds again; the difference of the flaxseeds amount filled before and after the addition of the bread corresponds to the volume of the bread. The measurements were

performed as duplicates to exclude inaccurate volume determinations due to the existence of air beneath the breads.

2.1.7 Determination of glutathione reductase activity

GshR activity in cell-free extracts was measured at 25ºC by monitoring the oxidation of NADPH in the reaction mixture (1 ml) at 340 nm. The reaction mixture contained 640 µl sodium phosphate buffer (143 mM NaH_2PO_4, 143 mM Na_2HPO_4, mixture 1:1 + 5 mM EDTA), 120 µl GSSG (10 mM), 100 µl NADPH (1 mM) and 100 µl extracts. The reduced GSH-content was measured with DTNB by adding 20 µl of reagent B as described under 2.1.5. The glutathione reductase activity was calculated with reference to controls that were incubated without addition of crude cellular extract.

2.1.8 Inactivation of the glutathione reductase from *Lactobacillus sanfranciscensis* TMW1.53

For insertional inactivation of the glutathione reductase gene, a 765-bp fragment of the glutathione reductase gene was obtained with PCR using primers gshknockV and gshknockR (chapter 4.1, table 21), carrying *Bam*HI restriction sites. Digestion and ligation into the *Bam*HI restriction site of plasmid pME-1 resulted in the non-replicating integration vector pME-1Δ*gshR*, which was cloned in *E. coli* DH5α and isolated with the Wizard Plus SV Minipreps DNA. For preparation of electro competent cells of *L. sanfranciscensis*, the strain was grown on mMRS medium supplemented with 1 % (w/v) glycine to an optical density (590 nm) of 0.7. The cells were harvested by centrifugation at 4°C (4000 x g, 15 minutes) and washed four times with 50 mL of 10 mM $MgCl_2$ solution, once with glycerol (10% v/v) and once with glycerol-sucrose solution (10% v/v, 0.5 M). Cells were resuspended in glycerol-sucrose solution and stored at –80°C in 100 µL aliquots. All washing and storage solutions were cooled on ice.

After electroporation, cells were incubated in mMRS broth at 30°C for 3 hours prior to plating on mMRS with 10 ppm erythromycin. To verify the insertion of plasmid pME-1Δ*gshR* into the glutathione reductase gene in cells from erythromycin-resistant colonies, PCR was carried out with primers targeting the regions upstream and downstream of the glutathione reductase gene (GTDHV and GTDHR, respectively) and the plasmid borne regions from pME-1 (eryV and T7, chapter 4.1, table 21). PCR products obtained with primers T7/GTDHV and GTDHR/eryV were sequenced. A scheme of the integration approach is given in chapter 4.2.3, figure 39 of this thesis.

2.1.9 Activity-staining of glutathione-reductase using SDS-page gel

Activity staining of GshR was performed after separation of crude cellular extracts on 12% SDS-polyacrylamide gels (16). The gel was immersed and shaken twice for 10 min in 25% v/v isopropanol in 10 mM Tris-HCl buffer (pH 7.9) to remove the SDS, and then finally equilibrated for renaturation in 50 mM Tris-HCl buffer (pH 7.9) for 15 minutes. The gel was soaked in the substrate solution (25 ml 50mM Tris-HCl buffer, pH 7.9, containing 4.0 mM GSSG, 1.5 mM β-NADPH, and 2 mM DTNB) with gentle shaking for 20 min. After a brief rinse with 50 mM Tris-HCl buffer (pH 7.9), the GshR activity was negatively stained in darkness by 50 ml 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and 1.6 mM PMS (phenazine methosulfate) for 10 minutes at ambient temperature. A clear zone against the blue background indicated GshR activity.

2.1.10 Protein analysis using RP-HPLC

The analyses were performed at the Hans-Dieter-Belitz-Institute, Freising, with the help of Dr. Herbert Wieser (see preface).

The crude protein content was determined by the Dumas method using an FP-328 combustion instrument. For the extraction of Osborne fractions, flour and lyophilized doughs (100 mg each) were extracted stepwise with 0.4 mol/L NaCl supplemented with 0.067 mol/L $HKNaPO_4$, pH 7.6 (2 x 1.0 mL) at 20°C (albumins/globulins), with 60% (v/v) ethanol (3 x 0.5 mL) at 20°C (gliadins) and with 50% (v/v) 1-propanol containing 2 mol/L urea, dithioerythritol (1%, w/v) and Tris-HCl (0.05 mol/L, pH 7.5) under nitrogen at 60°C (glutenin subunits). After centrifugation, the corresponding supernatants were combined and diluted to 2.0 mL with the respective extraction solvent. For the determination of SDS-soluble and SDS-insoluble proteins, flour and lyophilized doughs (100 mg) were extracted stepwise with 1% (w/v) SDS supplemented with 0.05 mol/L sodium phosphate pH 6.9 (2 x 1.0 mL) at 20°C. After centrifugation, supernatants were combined and lyophilized. Both dried extract and residue were dissolved in 50% (v/v) 2-propanol + 1% (w/v) + Tris-HCl (0.08 mol/L, pH 8.0) under nitrogen and magnetically stirred at 60°C for 20 minutes. All fractions were filtered through a 0.45 µm membrane. RP-HPLC was performed by using an instrument with a solvent module 126 and “System Gold” software. Aliquots of fractions were injected, separated and quantified on a Nucleosil 300-5-C8 column at 50°C by using a solvent gradient of increasing acetonitrile concentration in the presence of 1% (v/v) trifluoroacetic acid. The flow rate was 1.0 mL/min and the detection wavelength was 210 nm (40).

2.2 RESULTS

2.2.1 Biochemical characterization of the reference doughs

The determination of the requested metabolic activity of the fermentation starter cultures in the doughs was performed in respect to total plate count, pH-value, and the concentration of organic acids. Dough sample was analyzed from three different time-points: 0 hours (non-acidified dough); 5 hours (correspondent to a "Vollsauer" with exponentially grown lactic acid bacteria); and 24 hours (correspondent to a "Grundsauer" with a pH-value of 3.5). The chemically acidified dough was leavened with an addition of lactate / acetate (ratio 4:1). The plate count of the acidified dough showed no contamination growth.

Table 4. Microbiological characterization of sourdough prepared with „Tommi" wheat flour (type 550, A wheat)

Starter culture	Time (Hours)	Plate count (cfu[a] / g dough)	pH	Lactate[b]	Acetate[b]	Fermentation quotient[c]
chem. acidified [e]	0	$< 10^4$	6.35 ± 0.01	n.d.[d]	n.d.	--
	5	$< 10^4$	3.48 ± 0.03	41.0 ± 0.1	20.1 ± 2.1	2.0
	24	$< 10^4$	2.79 ± 0.04	179.3 ± 9.0	60.6 ± 4.9	3.0
L. sakei	0	$(1.0 \pm 0.1) \times 10^7$	6.21 ± 0.0	n.d.	n.d.	--
	5	$(1.25 \pm 0.08 \times 10^8$	4.44 ± 0.02	18.8	n.d.	--
	24	$(3.27 \pm 0.40) \times 10^9$	3.61 ± 0.04	52.6 ± 2.3	n.d.	--
L. sanfranciscensis	0	0.5×10^5	6.27 ± 0.01	n.d.	n.d.	
	5	$(4.3 \pm 1.8) \times 10^7$	5.54 ± 0.04	3.1 ± 0.2	1.4 ± 0.0	2.1
	24	$(3.2 \pm 0.2) \times 10^8$	3.62 ± 0.01	65.2 ± 0.1	8.2 ± 0.0	8.0
L. plantarum	0	$(5.7 \pm 1.1) \times 10^6$	6.22 ± 0.01	n.d.	n.d.	--
	5	$(11.1 \pm 9.3) \times 10^8$	4.22 ± 0.21	20.4 ± 0.4	n.d.	(51.0)
	24	$(1.0 \pm 0.4) \times 10^9$	3.34 ± 0.04	59.2 ± 7.0	0.1 ± 0.1	(592.0)

[a]cfu: colony forming unit, [b]concentrations in mmol / kg dough; [c]Fermentation quotient: lactate / acetate ratio, [d]n.d.: below detection limit, [e]reduced amount of acids for consecutively doughs, see text.

In all three fermented doughs total plate counts of $> 10^8$ cfu/g dough were detected after 24 hours of fermentation. Final pH-values of < 4.0 after 24 hours of fermentation were determined, corresponding to a "Vollsauer". *Lactobacillus sanfranciscensis* and *Lactobacillus plantarum* accumulated almost equal amounts of lactate after 24 hours of fermentation, 59.2 and 65.2 mmol/ kg dough, respectively. *L. plantarum* and *L. sakei* formed marginal amounts of acetate; these organisms utilize the Embden-Meyerhoff-Shunt almost exclusively for energy metabolism during wheat sourdough fermentations. *L. sanfranciscensis* accumulated higher amounts of acetate as expected, resulted in a lower fermentation quotient. The fermentation quotient describes the lactate/acetate ratio inside the fermentation system. Acetate plays a major role in determining bread aroma; the fermentation quotient is often used to describe the dough quality. Usually, the fermentation quotient lies within the range of 2.0–

3.5 in sourdoughs fermented with obligate heterofermentative lactobacilli. All three starter cultures, *L. plantarum*, *L. sakei*, and *L. sanfranciscensis* accumulated lesser amounts of organic acids during the fermentation period compared to the chemically acidified dough. In further approaches the supplemented amount of acids to the dough was therefore reduced. The sourdough fermentations resulted in a high reproducibility; minor standard deviations occurred between mean averages of two separately performed sourdough fermentations.

For evaluation of different degradation processes during wheat sourdough fermentations, also B-wheat and E-wheat besides A-wheat were applied in this study. The following tables summarise the data set for a "Bussard"-wheat flour and a "Dekan"-wheat flour.

Table 5. Microbiological characterization of sourdoughs prepared with „Bussard"-wheat flour (type 550, E wheat)

Starter culture	Time (h)	Plate count (cfu[a] / g dough)	pH	Lactate[b]	Acetate[b]	Fermentation quotient[c]	Amino-N[b]
chem. acidified	0	$< 10^4$	6.2 ± 0.14	n.d.[d]	n.d.	--	n.e.[e]
	5	$< 10^4$	3.75 ± 0.09	53.0 ± 1.5	12.8 ± 3.2	4.1	13.9 ± 2.7
	24	$< 10^4$	3.15 ± 0.08	150.3 ± 13.8	31.1 ± 2.4	4.8	18.2 ± 3.7
L. sanfranciscensis	0	n.e.	6.06 ± 0.04	n.d.	n.d.	--	5.2 ± 1.8
	5	$(1.5 \pm 0.14) \times 10^7$	4.75 ± 0.08	18.0 ± 1.3	3.6 ± 0.5	5.0	12.6 ± 3.4
	24	$(2.4 \pm 0.8) \times 10^8$	3.5 ± 0.0	76.0 ± 0.9	13.8 ± 0.7	5.5	18.3 ± 6.0
L. plantarum	0	$(5.5 \pm 0.9) \times 10^8$	6.09 ± 0.01	n.d.	n.d.	--	3.3 ± 1.4
	5	n.e.	3.75 ± 0.08	50.0 ± 3.0	n.d.	--	9.0 ± 3.4
	24	$(5.7 \pm 2.9) \times 10^8$	3.3 ± 0.0	102.8 ± 11.3	n.d.	--	9.3 ± 1.2

[a]cfu: colony forming unit, [b]concentrations in mmol / kg dough; [c]Fermentation quotient: Lactate / Acetate; [d]n.d.: below detection limit; [e]not evaluated

Table 6. Microbiological characterization of sourdough prepared with „Dekan"-wheat flour (type 550, B wheat)

Starter culture	Time (h)	Plate count (cfu[a] / g dough)	pH	Lactate[b]	Acetate[b]	Fermentation quotient[c]	Amino-N[b]
chem. acidified	0	$< 10^4$	6.25 ± 0.07	n.d.	n.d.	--	6.5 ± 1.1
	5	$< 10^4$	3.7 ± 0.0	43.2 ± 12.0	11.7 ± 1.2	3.7	7.2 ± 1.6
	24	$< 10^4$	3.25 ± 0.07	111.3 ± 0.5	28.5 ± 4.1	3.9	12.2 ± 3.3
L. sanfranciscensis	0	n.e.[e]	6.09 ± 0.03	n.d.	n.d.	--	6.3 ± 0.4
	5	$(5.9 \pm 0.4) \times 10^7$	3.65 ± 0.07	20.7 ± 0.9	4.8 ± 0.2	4.3	11.2 ± 2.2
	24	$(6.0 \pm 0.8) \times 10^7$	3.5 ± 0.0	79.1 ± 1.8	16.9 ± 0.5	4.7	16.9 ± 2.2
L. plantarum	0	$(4.5 \pm 1.7) \times 10^8$	6.06 ± 0.05	n.d.	n.d.	--	5.4 ± 1.4
	5	$(1.0 \pm 0.3) \times 10^9$	3.7 ± 0.0	56.5 ± 0.7	n.d.	--	10.4 ± 3.9
	24	1.3×10^9	3.4 ± 0.0	100.2 ± 5.5	n.d.	--	13.1 ± 0.1

[a]cfu: colony forming unit, [b]concentrations in mmol / kg dough; [c]Fermentation quotient: Lactate / Acetate, [d]n.d.: below detection limit; [e] not evaluated.

The total plate counts in fermentations with *L. sanfranciscensis* and Dekan-wheat flour showed lower total plate counts of approximately 10^7 cfu/g dough after 24 hours of fermentation, while the accumulation of organic acids during fermentation and the pH drop showed amounts as expected. Thus, it could be assumed that the effectively counts were higher, and a mistake was conducted in respect to serial dilutions.

The degradation of flour protein was detected by an increase of low-molecular amino nitrogen. The increase of low-molecular amino nitrogen content in the fermentation doughs was comparable to the increase in acidified doughs (see figure 6). This fact is in accordance to other studies; the protein degradation during wheat sourdough fermentations is mainly caused by endogenous proteases of the flour (6, 20, 26).

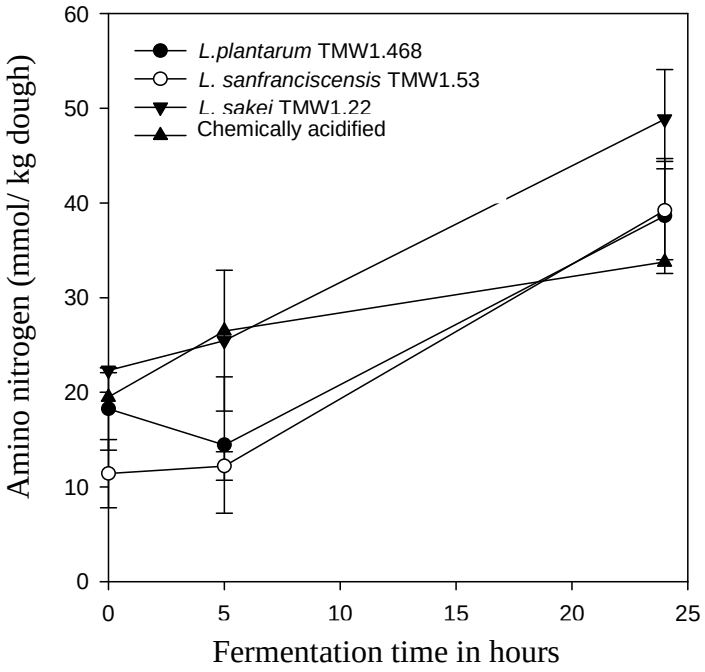

Figure 6. Free amino nitrogen content during sourdough fermentations with lactobacilli and chemically acidified dough.

Moreover, high-molecular proteins were extracted and evaluated using RP-HPLC. No differences could be seen on the quantitative evaluation of proteins after 5 hours of fermentation with the different lactobacilli strains in the Osborne fractions (albumins/globulins, gliadins, glutenin subunits). Only *Enterococcus faecalis* exhibited a proteolytic activity against the gluten proteins. The overall content of GMP was already significantly decreased after 5 hours of fermentations with the used strains. A drastically degradation of gluten proteins was observed after 24 hours of fermentation, whereas the glutenins and the GMP were more affected than the gliadins. The chemically acidified doughs and the fermented doughs with lactobacilli showed only marginal differences in respect to protein degradation. Thus, the endogenous flour proteases have the highest impact on protein degradation during wheat sourdough fermentations. The fermentations performed with the three different flour cultivars (“Tommi” A-wheat, “Bussard” E-wheat, and “Dekan” B-wheat) showed no significant trend related to protein degradation (data not shown, (40), and report AIF No. 14492 N).

2.2.2 Influence of sourdough dosage on the final bread volume

Sourdoughs were prepared and fermented for 20 hours at 30°C with *L. sanfranciscensis* TMW1.53, *L. plantarum* TMW1.468, and *L. sakei* TMW 1.22, subsequently 2%, 5%, 10%, and 20% of the fermented sourdoughs were added to final bread dough. In the case of *L. sanfranciscensis* sourdough, the baking trials were performed two-times with "Tommi"-wheat flour and once with commercially, ascorbic acid-free wheat flour. The baking trials with *L. plantarum* and *L. sakei* sourdough were performed only two-times with "Tommi"-wheat flour (data not shown). No significant differences were observed in the final bread volume concerning the sourdough dosage in percentages, or the applied flours (data not shown).

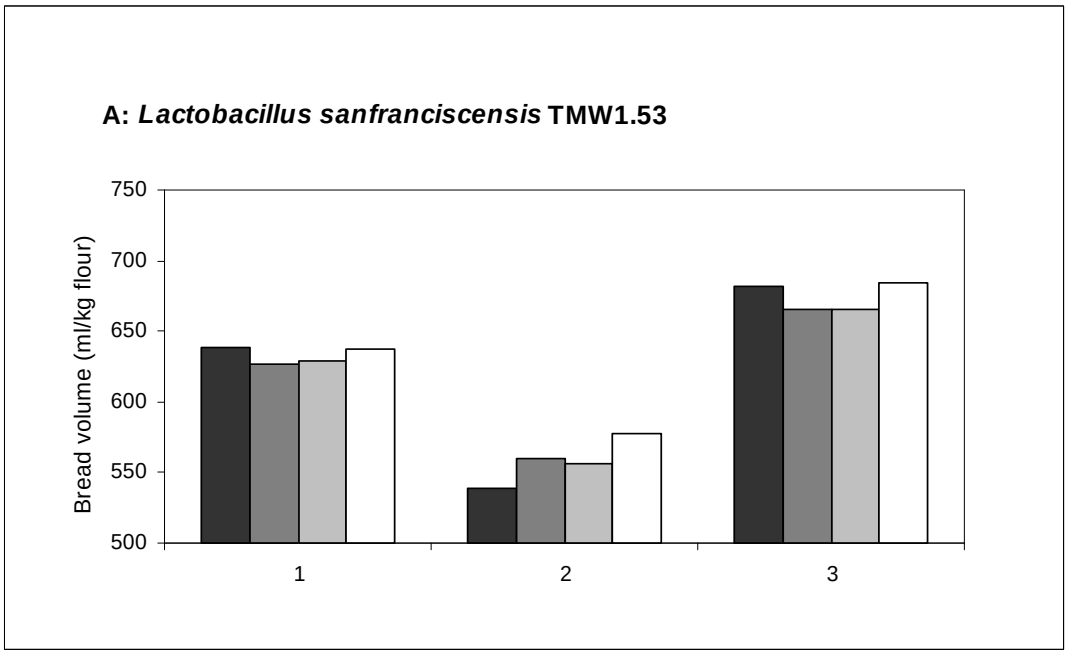

Figure 7. Influence of different sourdough dosages on the bread volume at three baking days. Sourdoughs were prepared at different days with Tommi-wheat and commercially available flour (1: Tommi, day 1; 2: Tommi, day 2; 3: commercially available flour, day 3). Sourdoughs (TA 200) were inoculated with *L. sanfranciscensis* TMW1.53 (A), and fermented for 20 hours at 30°C. Black bar = 2% sourdough; dark-grey bar = 5% sourdough; grey bar = 10% sourdough; and white bar = 20% sourdough dosage.

The final bread volumes showed no differences in respect to the diverse starter cultures: *Lactobacillus sanfranciscensis* (obligate heterofermentative), *Lactobacillus plantarum* (facultative heterofermentative), and *Lactobacillus sakei* (facultative heterofermentative). Nonetheless, significant differences were observed in bread volumes between two baking days with the same final dough process and fermentation organism (e.g. A: *Lactobacillus sanfranciscensis* 1, 2).

2.2.3 Influence of organic acids on the final bread volume

The bread volume is essentially dependent on the dough pH-value; therefore, the influence of organic acids was determined on the final bread volume. Primarily, lactate and acetate amounts were introduced into doughs comparable to *in vivo* accumulated amounts of these acids. The organic acids were supplemented at the beginning of the fermentation. A final dosage of 10% sourdough was added to the final bread doughs. All sourdoughs were prepared without the addition of viable microorganisms.

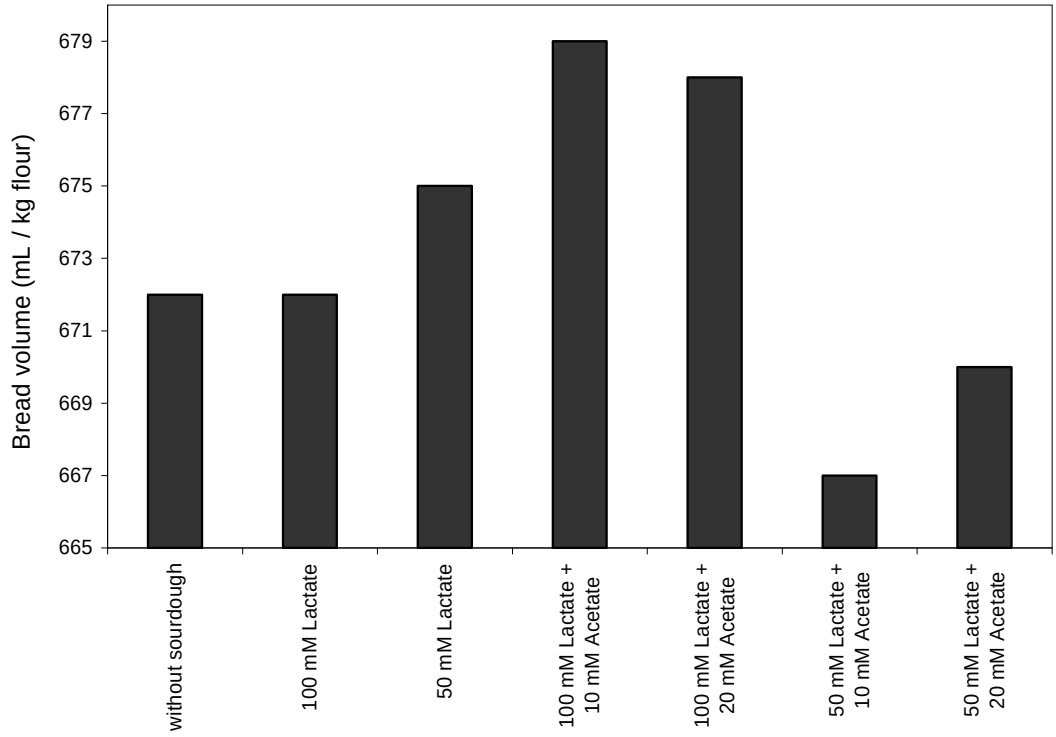

Figure 8. Final bread volume of sourdough prepared breads with a dosage of 10%. The sourdoughs were prepared without supplementation of microorganisms, only organic acids were added.

The differences were marginal between the final bread volumes; the mean value was 673.3 ml/kg flour, the standard variation 4.3 ml/kg flour. The lowest and maximum bread volume showed only a difference of 12 ml/kg flour. Therefore, the generally accumulated organic acids during wheat sourdough fermentations with lactobacilli are having no impact on the final bread volume. However, it should be noticed, that the effect of acids depends on the buffering capacity of the system.

Consecutively, the pH-value was adjusted to 3.6 with organic acids in sourdough fermentations; in pre-trials the amount of acids / g flour was determined to reach a specific pH-value. For the baking trials sourdoughs were fermented with the addition of lactic- and

acetic acid, moreover also a mixture of both acids. Furthermore, sourdoughs were prepared with hydrochloric acid; this approach served to distinguish between pH-value effects and influences of specific acids. Significant differences in final bread volume were observed between two baking days for bread doughs produced with a constant process (see figure 7).

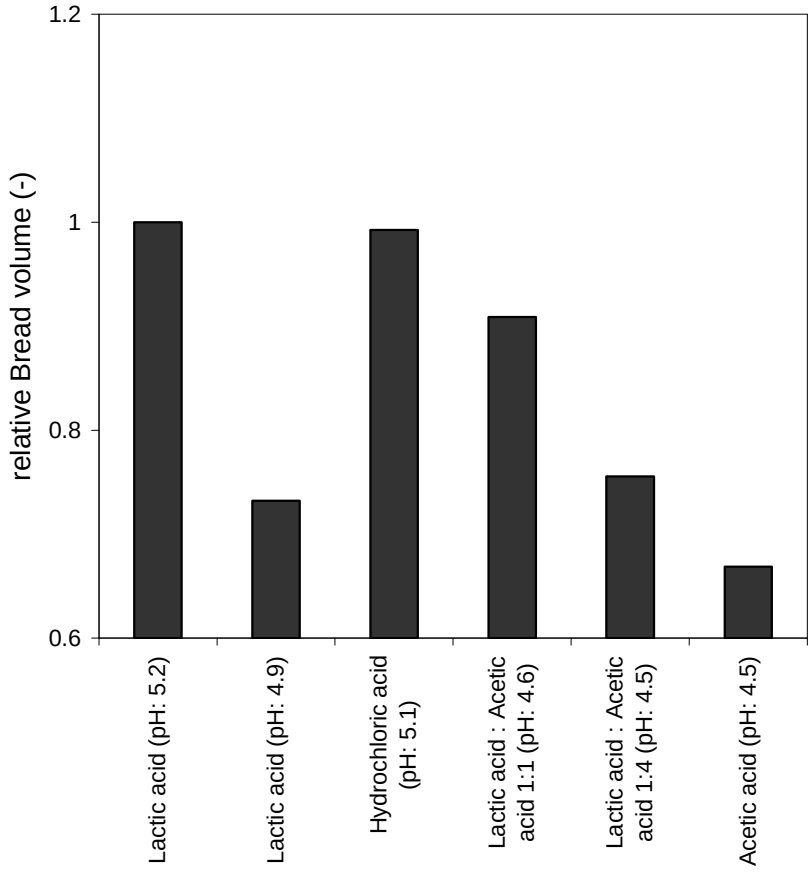

Figure 9. Volume of sourdough prepared breads (Bread dough pH). The pH-values of sourdoughs were exactly adjusted to pH 3.6 with different organic acids. The sourdough dosage was 10% of the final bread dough.

Hence, every day one reference bread was baked with a dosage of 10% sourdough (pH: 3.6) added to the final bread dough. The relative volumes of the baked breads were determined according to following formula: volume $_{\text{bread xy}}$ / volume $_{\text{reference bread}}$.

The relative final bread volume of the lactic acid bread is by definition 1. No significant difference was observed between lactic acid and hydrochloric acid acidified breads. A distinct decrease of the final bread volume was observed by the addition of acetic acid (last bar). The final pH-values of the bread doughs prepared with lactic acid sourdoughs, and hydrochloric acid sourdoughs were 5.2 and 5.1, respectively. The breads produced with acetic acid acidified sourdough showed a pH-value of 4.5. The bread volume collapsed by the addition of triple amounts of lactic acid (second bar). The final bread volume was even lower than the

bread volumes prepared with a 1:1 ratio or 1:4 ratio of lactate to acetate, respectively. Notwithstanding, the pH-values of the latter breads were clearly decreased with 4.6 and 4.5 compared to a bread dough pH-value of 4.9. Therefore, it is proposed that not only the pH-value determines the final bread volume, but also the subsequent parameters (see figure 10). The baker's yeast could be inhibited by specific amounts of acetic acid.

A **B**

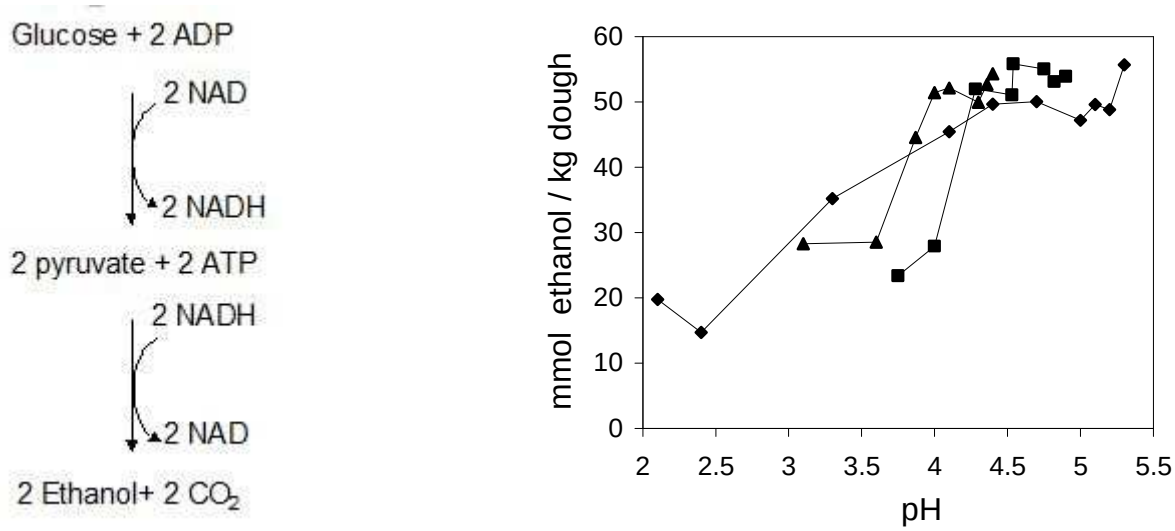

Figure 10. (A): Simplified presentation of yeast fermentation. (B): Accumulation of ethanol (mmol) of baker's yeast in chemically acidified doughs after two hours of fermentation. The doughs were adjusted to the requested pH-value with hydrochloric acid (filled diamond), acetic acid (filled cube), and lactic acid (filled triangle).

The fermentation efficiency of the baker's yeast was determined in following approach to confirm the inhibitory effect of acetic acid. Figure 10 A summarizes the fact, that there is a direct link between accumulation of ethanol and gas formation by yeast under anaerobic conditions. The relative amount of ethanol accumulated by baker's yeast in different doughs acidified with lactic acid, acetic acid, and hydrochloric acid is shown in figure 10 B, the ethanol concentration was measured after two hours of fermentation in a liquid dough with a dough yield of 400 (TA 400). The specific influence of acetic acid supplementation independent to the pH-value could be clearly seen. Thus, the presence of acetic acid has an inhibitory effect of the baker's yeast during fermentation.

Moreover, the final bread volume was compared with *L. sanfranciscensis* sourdough dosage to breads with *L. sanfranciscensis* sourdough dosage supplemented with fructose. Again it should be proven, that acetic acid accumulation during sourdough fermentations can inhibit the baker's yeast. *L. sanfranciscensis* exclusively formed acetic acid in fermentations; no

accumulation of ethanol was observed (data not shown) by the addition of fructose (5% from the initial flour weight). The sourdoughs were fermented for 48 hours to gain the highest acetic acid concentrations. The final bread volumes of breads prepared with the addition of fructose were slightly decreased in volume in comparison with the reference bread (see figure 11). The reduction in volume could be caused by higher concentrations of acetic acid in the doughs, certainly also the presence of fructose could affect dough characteristics.

Figure 11. Influence of supplemented fructose to sourdoughs fermented with *L. sanfranciscensis* TMW1.53 on the final bread volume.

2.2.4 Influence of the used starter culture in sourdough fermentations on the final bread volume

The effect of starter culture selection for sourdough fermentations upon bread volume was determined with five different microorganisms. Obligate heterofermentative and facultative heterofermentative species were chosen as well as *Enterococcus faecalis* which clearly contributed to gluten proteolysis in sourdough fermentations (40). Sourdoughs were fermented with: *L. sanfranciscensis* TMW1.53, *L. plantarum* TMW1.468, *L. sakei* TMW1.22, *L. fermentum* TMW1.890, and *Enterococcus faecalis* TMW2.22. The sourdoughs were prepared with a dough yield of 200 (TA 200) and fermented for approx. 20 hours at 30°C; the final bread doughs contained 10% sourdough dosage. The baking approaches were performed as duplicates at three independent baking days. Common wheat flour (free of ascorbic acid) was used in this approach compared to the genuine flour used in chapter 2.2.2. No specific strain could be selected to gain an increase in final bread volume (see figure 12).

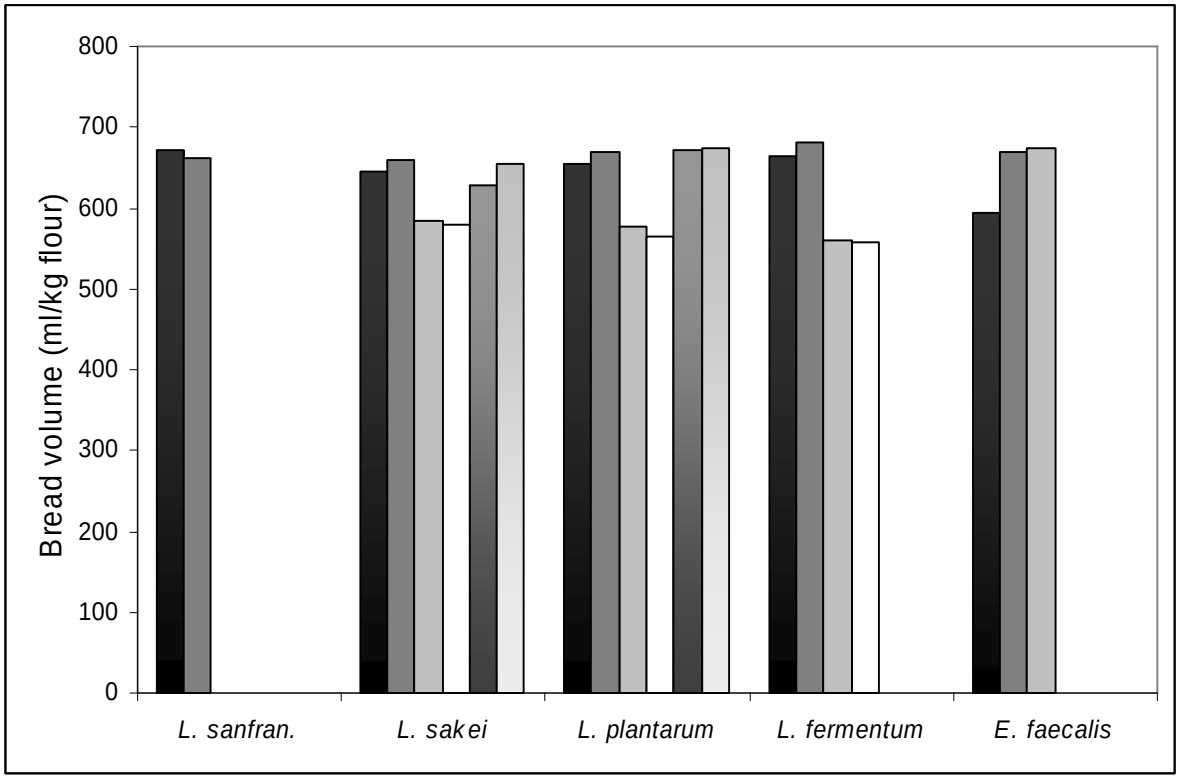

Figure 12. Influence of the used micro organisms in sourdough fermentations on the final bread volume. The baking approaches were conducted at several baking days. The different colored bars represent independent baking days. Used strains: *L. sanfranciscensis* TMW1.53, *L. sakei* TMW1.22, *L. plantarum* TMW1.468, *L. fermentum* TMW1.890, and *E. faecalis* TMW2.22.

The assumption that reductive, obligate heterofermentative strains like *L. sanfranciscensis* produce breads with a decreased volume in comparison with H_2O_2 generating strains, e.g. *L. sakei* was not observed. The selected sourdough dosage of 10% to the final bread dough resulted in insufficient results in respect to classification of strains based on their generated bread volume. The reproducibility of bread volumes were sufficient between breads baked on the same day with the same fermentation strain. All pre-cultures were checked for purity using microscopy before inoculation of the respective sourdough; therefore, a contamination or development of spontaneous flora was very unlikely. The complexity of the fermentation and baking process avoided a classification in respect to bread volume based on different used starter cultures. Moreover, the different physiological condition of the added baker's yeast must be mentioned on the diverse baking days. The influence of dry yeast usage was determined to compensate the different physiological conditions of the baker's yeast; this approach provided insufficient results due to non-activity of the dry yeast in fermentations (type: Farmipan Red).

Figure 13. Appearance of baked breads with dry yeast
(left bread) and reference bread with fresh yeast (right bread)

The breads baked with dry yeast showed a quarter of the volumes in comparison with breads baked with fresh baker's yeast; this could already be seen in the dough stage, after preparation of the doughs included dough rest only a rarely yeast performance and dough development was observed (see figure 13).

2.2.5 Influence of the sourdough fermentation period on the bread volume

The bread volume of an overnight fermented sourdough with lactic acid was compared with a freshly prepared sourdough. The breads produced from the freshly prepared sourdough were decreased 2% in volume compared with the overnight fermented sourdough (662 vs. 677 ml/ kg flour). In another trial, sourdough was fermented with lactic acid for 20 hours and 70 hours, respectively. The final bread volume of the 20 hours fermented sourdough was 646 ml/kg flour, whereas the bread volume of the 70 hours fermented sourdough was 513 ml/kg flour; thus, a 21% decrease in final bread volume was observed for the 70 hours fermented sourdough. The repetition of the baking trial confirmed the above mentioned result, but the decrease of final bread volume was not so pronounced (only 3% in comparison with 21% in the first approach). The difference of 18% in decrease of bread volume could not be clearly clarified between the two approaches. Microbiological contaminations of the doughs could be excluded due to chemical acidification.

2.2.6 Influence of ascorbic acid addition on the volume of sourdough breads

The fundamental difference between the above mentioned approaches and the real baking process in bakeries is the usage of ascorbic acid. Ascorbic acid is the main added baking improver with stabilization effects upon the gluten network:

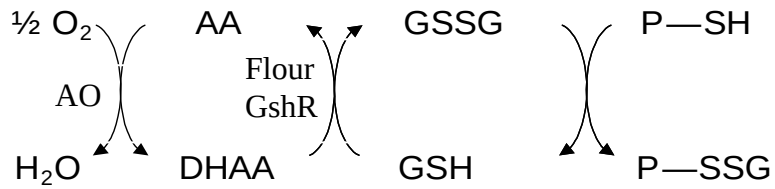

Figure 14. Oxidation of glutathione by endogenous flour glutathione dehydrogenase contributes to stabilization of the gluten network in wheat doughs (according to Hahn and Grosch, (15, 37)). AA: ascorbic acid, DHAA: dehydroascorbic acid, AO: ascorbic acid oxidase, GshR: glutathione dehydrogenase, P: protein

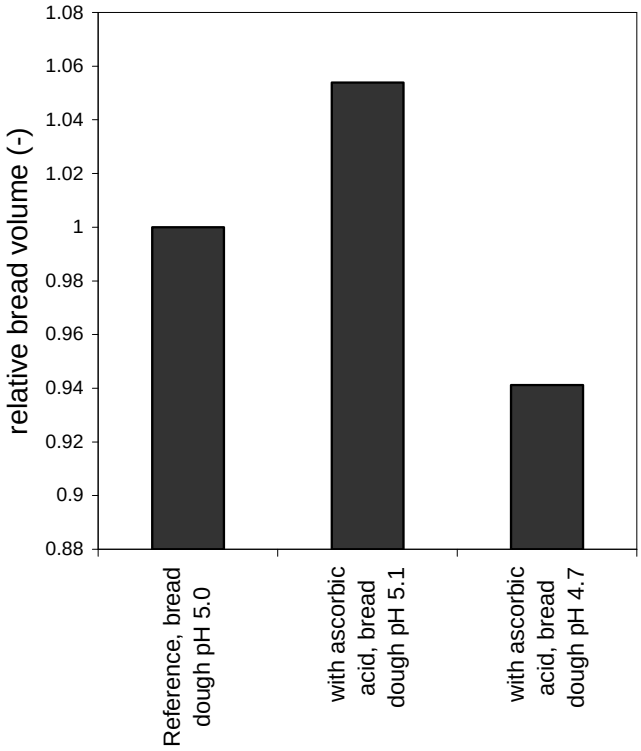

Figure 15. The influence of ascorbic acid towards bread volume is pH-dependent.

The influence of the final bread dough pH-value on the effect of ascorbic acid was determined. Reference bread was baked with a lactic acid sourdough dosage of 10%; ascorbic acid in a common concentration of 0.02% in relation to initial flour weight was supplemented to the sourdough, though the dilution of the sourdough in the final bread dough was considered. The final pH-value was not adjusted after addition of ascorbic acid to the first sourdough. In the second sourdough, ascorbic acid was dissolved in water and the pH-value was adjusted using sodium hydroxide to 7.2. The final pH-values of the baked breads differed by 0.4 pH-units. The increase in bread volume due to addition of ascorbic acid was only observed in breads baked with higher pH-values of the dough, e.g. pH-value of 5.1.

2.2.7 Influence of microbiological catalysed redox reactions on bread volume

L. sanfranciscensis TMW1.53 possesses a glutathione reductase, its activity caused an increase of thiol-groups during wheat sourdough fermentations (see also chapter 2.2.8). Other lactobacilli, e.g. *L. sakei*, showed no glutathione reductase activity; thus, there was observed no increase in thiol-groups during wheat sourdough fermentations (37). The quantity of free thiol-groups plays a major role in development of the gluten network; hence, also a main impact on bread volume could be seen. Thus, a difference in final bread volume was expected using the different lactobacilli in sourdough fermentations.

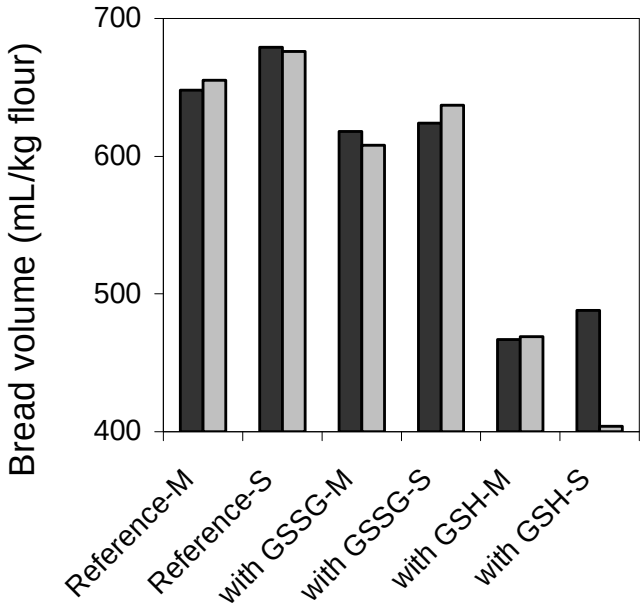

Figure 16. Influence of oxidized and reduced glutathione on the final bread volume. Glutathione was supplemented to sourdoughs acidified with lactic acid (-M) and to sourdoughs fermented with *L. sanfranciscensis* TMW1.53 (-S). The experiments were performed in duplicates, dark-grey and grey bars.

As shown in figure 12, no classification based on the selected starter cultures in sourdough fermentations in respect to the final bread volume could be established.

In addition, sourdoughs were fermented with GSSG (10 mM) and GSH (10 mM) supplemented at the beginning of fermentation; Following, the final bread volume was determined. *L. sanfranciscensis* sourdoughs and chemically acidified doughs were used in this approach. The bread volume was increased by 5% when 10% sourdough fermented with *L. sanfranciscensis* was added to the final bread dough in comparison to the reference bread. As expected, addition of the reduced form of glutathione (GSH) resulted in decreased final bread volumes. Thus, supplemented GSH degraded the protein network, and therefore contribute to

a reduction of the gas holding capacity of the gluten. The effect of glutathione addition on final bread volume was comparable between chemically acidified sourdoughs and sourdoughs fermented with *L. sanfranciscensis*. The expectation was that *L. sanfranciscensis* reduce GSSG to form GSH resulting in a final bread volume reduction in comparison to chemically acidified sourdoughs, but this was not the case. Thus, indicating that glutathione reductase activity from *L. sanfranciscensis* is playing just a minor role in respect to final bread volume; the influence of microbial glutathione reductase after addition of exogenous glutathione in sourdoughs on the final bread volume (10% sourdough dosage) was negligible.

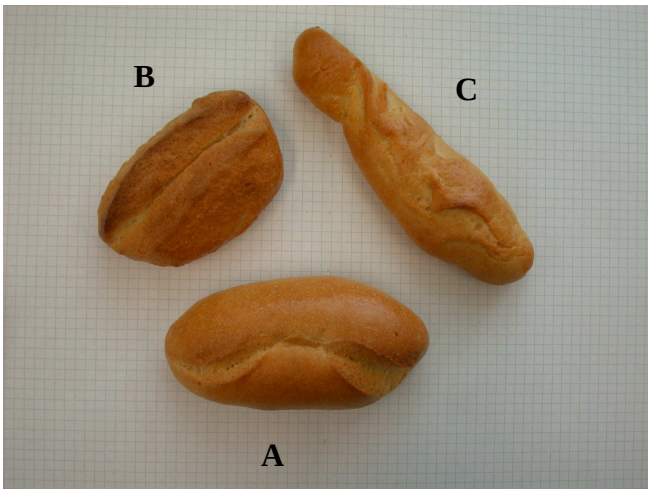

Figure 17. Appearance of breads in standard baking trials with *L. sanfranciscensis* TMW1.53 fermented sourdoughs: (A) Reference, (B) supplemented with 10 mM GSSG, (C) supplemented with 10 mM GSH.

Figure 17 shows the baked breads with supplemented GSSG and GSH, respectively. The reference bread was prepared with a dosage of 10% *L. sanfranciscensis*-sourdough (A), the baked bread with addition of oxidized glutathione to *L. sanfranciscensis*-sourdough showed already a decreased volume compared with the reference bread (B). *L. sanfranciscensis*-sourdoughs supplemented with reduced glutathione showed a significant decrease in final bread volume (C).

Oxidoreductases of lactobacilli could generate reduced thiol compounds during wheat sourdough fermentations. Low-molecular weight compounds are capable bread aroma precursors; however, the dough rheology could be affected by degradation of gluten proteins caused by a decrease of the redox potential in the dough system. Approaches in this study (see figure 20) indicated that obligate heterofermentative lactobacilli, e.g. *L. reuteri* and *L. pontis* exert comparable effects on thiol levels during wheat sourdough fermentations as *L.*

sanfranciscensis. Strains of these species and other lactobacilli were screened for glutathione reductase activity on biochemical and genetically levels to establish whether their effects on thiol exchange reactions are also attributable to glutathione reductase activities. Moreover, *L. reuteri* BR11 and the cognate mutant strain *L. reuteri* BR11Δ*cyuC*, which is deficient in a cystine uptake system (25), were included in the analysis. Several lactobacilli DNA were screened in PCR by using the degenerated primers Deg/gshRV and Deg/gshRR (see chapter 4.1.1 and 5.1.10), respectively. *L. brevis* TMW 1.57, *L. plantarum* TMW 1.460, *L. johnsonii* TMW 1.192, *L. frumenti* TMW 1.635, *L. acidophilus* TMW 1.18, *L. hilgardii* TMW 1.45, and *L. pentosus* TMW 1.10 were found to harbor glutathione reductase genes related to *gshR* of *L. sanfranciscensis*; however, no amplification product was obtained with DNA from *L. reuteri* TMW 1.106, *L. pontis* TMW1.84 or *L. reuteri* BR11 (data not shown and Table 7).

Table 7. Occurrence of glutathione reductase genes and cystathionine-γ-lyase genes in different lactobacilli

Glutathione reductase sequences	**Cystathionine-γ-lyases sequences**
L. sanfranciscensis TMW1.53	*L. reuteri* TMW1.10
L. brevis TMW1.57	*L. reuteri* TMW1.976
L. plantarum TMW1.460	*L. panis* TMW1.648
L. johnsonii TMW1.192	*L. fermentum* TMW1.890
L. frumenti TMW1.635	*L. mindensis* TMW1.1206
L. acidophilus TMW1.18	*L. pontis* TMW1.84
L. hilgardii TMW1.45	
L. pentosus TMW1.10	

The absence of glutathione reductase activity in *L. pontis* and *L. reuteri* was verified by determination of the activity in crude cellular extracts after separation on SDS-PAGE gels (Figure 18). Strains without glutathione reductase activity exhibited cystathionine-γ-lyase (CgL) activity (Table 8).

Based on the partial sequence of the cystathionine-γ-lyase of *L. fermentum* TMW1.890, the whole sequence of the gene was completed using inverse PCR (see chapter 5.2 Sequences). A knockout-mutant of the glutathione reductase (gshR) from *L. sanfranciscensis* TMW1.53 was designed according to chapter 2.1.8; the influence of the glutathione reductase was evaluated with this approach on thiol-levels during wheat sourdough fermentations. In addition, following lactobacilli strains were examined for glutathione reductase activity according to

chapter 2.1.7: *L. sanfranciscensis* TMW1.53, *L. sanfranciscensis* TMW1.53Δ*gshR*, *L. pontis* TMW1.84, *L. reuteri* BR11, and a cognate mutant of *L. reuteri* BR11 unable to transport cystine (*L. reuteri* BR11Δ*bsp*A). *L. sanfranciscensis* showed a glutathione reductase activity of 45 nmol/min/mg, while the activity of the cognate mutant strain *L. sanfranciscensis* TMW1.53Δ*gshR* was strongly reduced to 14 nmol/min/mg. No glutathione reductase activity was observed for *L. pontis* TMW1.84 and *L. reuteri* BR11.

Table 8. Glutathione reductase and cystathionine-γ-lyase activity in sourdough lactobacilli.

	L. sanfrancscensis TMW1.53	*L. sanfranciscensis* TMW1.53Δ*gshR*	*L. reuteri* BR11	*L. reuteri* BR11 Δ*cyuC*	*L. pontis* TMW1.84
GshR activity[a]	45	14	-	-	-
gshR[b]	+	-	-	-	-
CgL activity	-	-	+	+	+
CgL, PCR	-[c]	n.d.	+[d]	n.d.	+[c]

a) glutathione reductase activity in crude cellular extract, nmol min^{-1} mg^{-1}, - indicates the absence of GshR activity in crude cellular extracts after separation on SDS-PAGE. b) Detection of a glutathione reductase gene using the degenerated primers Deg-gshRV and Deg-gshRR. c) (38). d) (35).

The results were supported by activity staining for glutathione reductase using SDS-gel according to chapter 2.1.9.

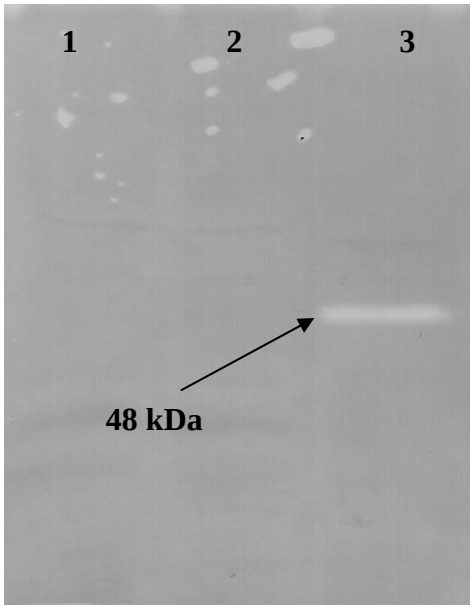

Figure 18. Detection of glutathione reductase acivity in crude cellular extracts of *L. reuteri* BR11 (Lane 1), *L. pontis* TMW1.84 (Lane 2) and *L. sanfranciscensis* TMW1.53 (Lane 3) after separation of crude cellular extracts by SDS-PAGE. The location of GshR with a predicted relative molecular weight (M_w) of 48.614 is indicated.

2.2.8 Thiol levels in sourdough fermented with *L. sanfranciscensis* TMW1.53 and *L. sanfransiscensis* TMW1.53Δ*gshR*

To determine whether GshR is involved in the reduction of thiol groups in wheat sourdoughs, thiol levels were quantified in SDS-extracts of wheat sourdoughs fermented with *L. sanfranciscensis* TMW1.53 and TMW1.53Δ*gshR*. 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. Chemically acidified dough was used as a control. Both strains grew to high cell counts ($7.0 \pm 0.5 \times 10^8$ cfu/g) after 24 hours of fermentation. The pH of doughs fermented with *L. sanfranciscensis* after 5 hours was 4.38 ± 0.05 and 3.45 ± 0.05 after 24 hours, respectively. Fermentation with *L. sanfranciscensis* increased thiol levels in dough (Figure 19). In chemically acidified doughs, thiol level decreased during fermentation. During fermentation with the *gshR*-mutant strain, the pH decreased to 4.32 ± 0.05 after 5 hours of fermentation and to pH 3.47 ± 0.05 after 24 hours. The thiol content of sourdoughs fermented with the *gshR*-mutant was comparable to that of chemically acidified doughs.

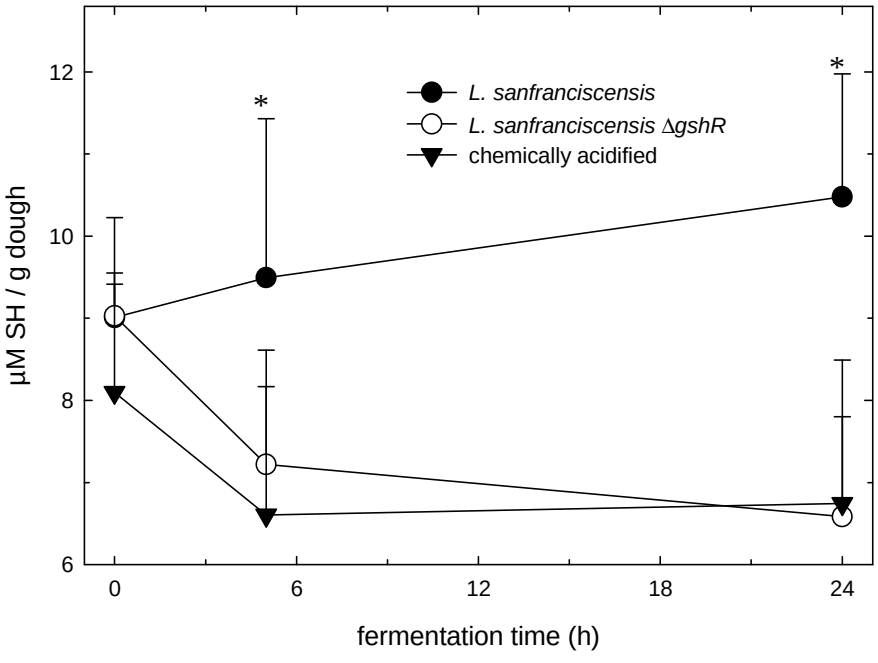

Figure 19. Thiol levels SDS-extracts from chemically acidified wheat doughs and dough fermented with *L. sanfranciscensis* TMW1.53 and *L. sanfranciscensis* TMW1.53Δ*gshR*. Shown are the means ± standard deviations from five independent experiments, data differing significantly ($p<0.05$) from chemically acidified doughs are marked with an asterisk.

2.2.9 Thiol levels in sourdough fermented with *L. reuteri* BR11, *L. reuteri* BR11Δ*cyuC* and *L. pontis* TMW1.84

The effects of glutathione reductase negative, heterofermentative lactobacilli on thiol levels in dough were determined with strains *L. pontis* TMW1.84 as well as *L. reuteri* BR11 and BR11Δ*cyuC*. All strains acidified wheat doughs to pH-values ranging from 3.20 to 3.32 after 24 hours of incubation. Remarkably, the effects of the glutathione-reductase negative strains on thiol levels in dough were qualitatively comparable to that of *L. sanfranciscensis* (Figure 19). Thiol levels in doughs fermented with *L. reuteri* BR11Δ*cyuC* were consistently higher than levels in doughs fermented with the corresponding wild type strain. The lack of a cystine uptake system in the mutant strain could explain the higher thiol levels in comparison to the wild type strain of *L. reuteri* BR11.

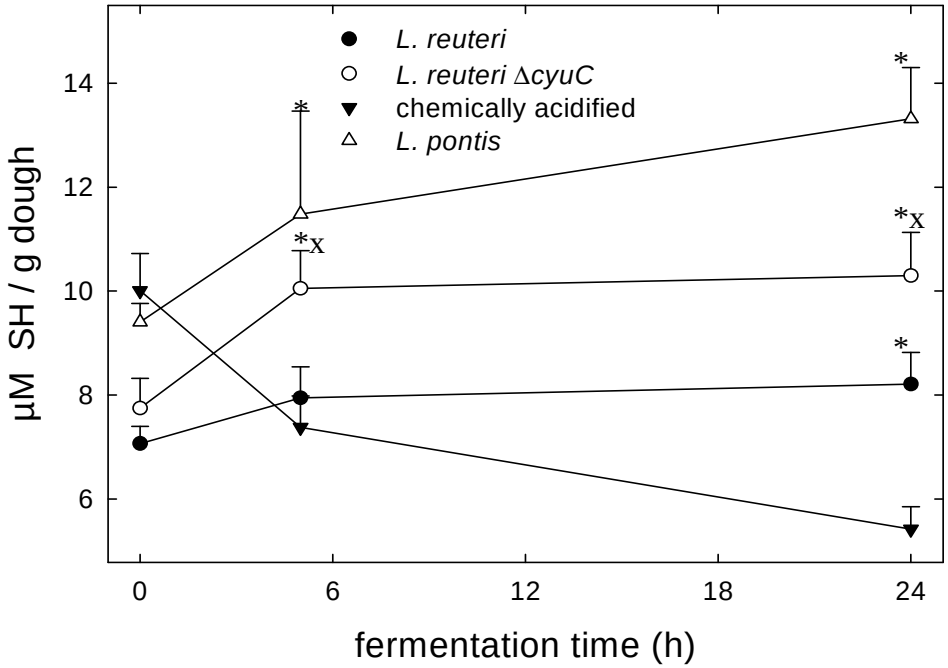

Figure 20. Thiol levels in sourdough fermented with *L. reuteri* BR11, *L. reuteri* BR11Δ*cyuC*, *L. pontis* TMW1.84 and chemically acidified doughs. Shown are the means ± standard deviations from three independent experiments, data differing significantly ($p<0.05$) from chemically acidified doughs are marked with an asterisk; significant differences ($p<0.05$) between doughs fermented with *L. reuteri* BR11 and BR11Δ*cyuC* are marked with an x.

Gluten has a prominent position during preparation of wheat dough, for the development of the gluten network the formation of inter- and intramolecular disulfide bonds is crucial between the glutenin subunits (39). Formation of gluten modifications during sourdough fermentations with lactobacilli affects dough rheology, texture and final bread volume of the wheat doughs. The degradation of the SDS-insoluble gluten macropolymer (GMP) to SDS-

soluble low-molecular weight compounds is supported by the proteolytic activity of lactobacilli, thus the gluten quality in wheat doughs with 10-20% sourdough dosage is adulterated in comparison with direct produced wheat doughs (7).

The influence on thiol levels during wheat sourdough fermentations with thermophilic, heterofermentative lactobacilli, e.g. *L. reuteri* and *L. pontis* is comparable with fermentations with *L. sanfranciscensis*; however, the indicated strains exhibited no glutathione reductase activity (27). The increased thiol levels in sourdoughs fermented with *L. reuteri* and *L. pontis* are attributed to other thiol active enzymes. Both strains harboring a cystathionine-γ-lyase gene and exhibited also cystathionine-γ-lyase activity in crude cellular extracts (see table 8). An influence of the active enzyme on thiol levels during wheat sourdough fermentations could be existent due to metabolism of cysteine and thiol exchange reactions.

2.2.10 Effects of *L. sanfranciscensis* TMW1.53 and cognate mutant TMW1.53Δ*gshR* on gluten proteins during sourdough fermentations

The use of a method combining extract and HPLC allowed a more detailed study on protein degradation (see chapter 2.1.10) during wheat sourdough fermentations. Dough proteins, here gliadins and glutenins were fractionated by a modified Osborne procedure according to Wieser et al. (40). Significant differences in the GLIA amounts could not be detected over a fermentation period of 24 hours with chemically acidified dough, sourdough fermented with *L. sanfranciscensis* TMW1.53, and the cognate mutant TMW1.53Δ*gshR*. However, a significant difference in the GLUT amounts was detected after 24 hours of fermentation with a decrease to 34% in *L. sanfranciscensis* TMW1.53 sourdoughs in comparison to a decrease to only 56% in TMW1.53Δ*gshR* sourdoughs.

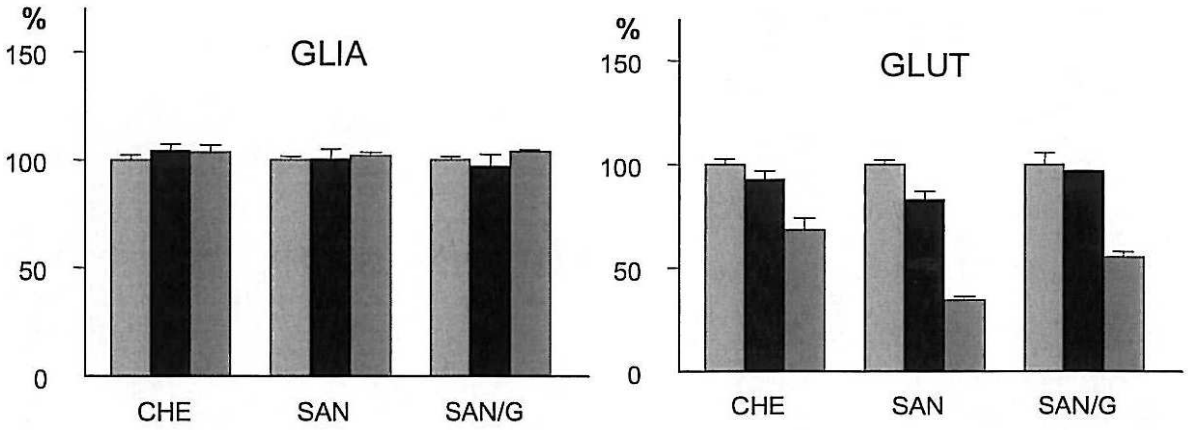

Figure 21. Relative amounts of gliadins (GLIA) and glutenins (GLUT) in sourdoughs after 0, 5, 24 hours of fermentation. CHE: chemically acidified dough, SAN: *L. sanfranciscensis* TMW1.53 sourdough, SAN/G: *L. sanfranciscensis* TMW1.53Δ*gshR* sourdough. 0 hours = 100 %.

Total plate counts of the two sourdoughs of *L. sanfranciscensis* TMW1.53 and the mutant exhibited similar results > 10^8 cfu/g dough after 24 hours of fermentation. The chemically acidified dough showed total plate counts < 10^4 cfu/g dough throughout the fermentation period indicating no contamination growth, e.g. spontaneous microbiota. The pH-value as well as the fermentation quotient showed similar results for *L. sanfranciscensis* sourdoughs and the mutant TMW1.53Δ*gshR* sourdoughs, respectively. The chemically acidified dough showed somehow increased organic acids values compared to the doughs fermented with microorganisms; although, they showed still values at the upper range of natural acidified sourdoughs. Therefore, a direct effect is shown of the glutathione reductase on glutenin proteins during wheat sourdough fermentations. The degradation of the glutenin protein fraction differs by approx. 20% between the wild type strain of *L. sanfranciscensis* and the cognate mutant.

Table 9. Microbiological characterization of sourdoughs prepared with commercially, ascorbic acid-free wheat flour for gluten protein determination.

Starter culture	Time (Hours)	Plate count (cfu[a] / g dough)	pH	Lactate[b]	Acetate[b]	Fermentation quotient[c]
chem. acidified	0	< 10^4	n.a.	n.d.[d]	n.d.	-
	5	< 10^4	n.a.	14.2	6.4	2.2
	24	< 10^4	n.a.	48.6	20.3	2.4
L. sanfranciscensis	0	3.0 x 10^5	5.62	n.d.	n.d.	-
TMW1.53	5	5.0 x 10^7	4.38	8.8	2.2	4.0
	24	7.4 x 10^8	3.45	35.9	6.1	5.9
L. sanfranciscensis	0	3.8 x 10^5	5.55	n.d.	n.d.	-
TMW1.53Δ*gshR*	5	4.6 x 10^7	4.32	4.8	0.6	8
	24	7.2 x 10^8	3.47	30.2	7.9	3.8

[a]cfu: colony forming unit, [b]concentrations in mmol / kg dough; [c]Fermentation quotient: lactate / acetate ratio, [d]n.d.: below detection limit, n.a.: not analyzed

The production of breads based on sourdoughs fermented with the mutant strain *L. sanfranciscensis* TMW1.53Δ*gshR* was not feasible due to the existence of a genetically modified organism. Therefore, the direct influence of the glutathione reductase on final bread volume could not be evaluated. Indeed, it should be considered that the sourdough content is approximately between 10-20% of the final bread dough. The impact of the enzyme would have been diminished by "dilution" of fresh prepared dough. Moreover, yeasts could equalize possible differences in the following baking process (see figure 5 – Rapid Mix Test).

2.2.11 Influence of "Vitalkleber" and Diacetyl-tartaric-acid-ester (DATEM) on the final bread volume

Vitalkleber and DATEM are the main improvers used by bakers beside ascorbic acid. The gluten quantity, hence bread volume is increased after addition of Vitalkleber; the ester serves as an emulsifier with stabilization effects on the existing gluten network. Both bakery improvers were provided by Mühlenchemie (Ahrensburg, Germany – "Vitalkleber": EMCEvit C and DATEM: Mulgaprime 16). The bakery improvers were added at the beginning of sourdough fermentations according to manufacturer's instructions (Vitalkleber: 2.0%/1000 g flour; DATEM: 0.3%/1000 g flour).

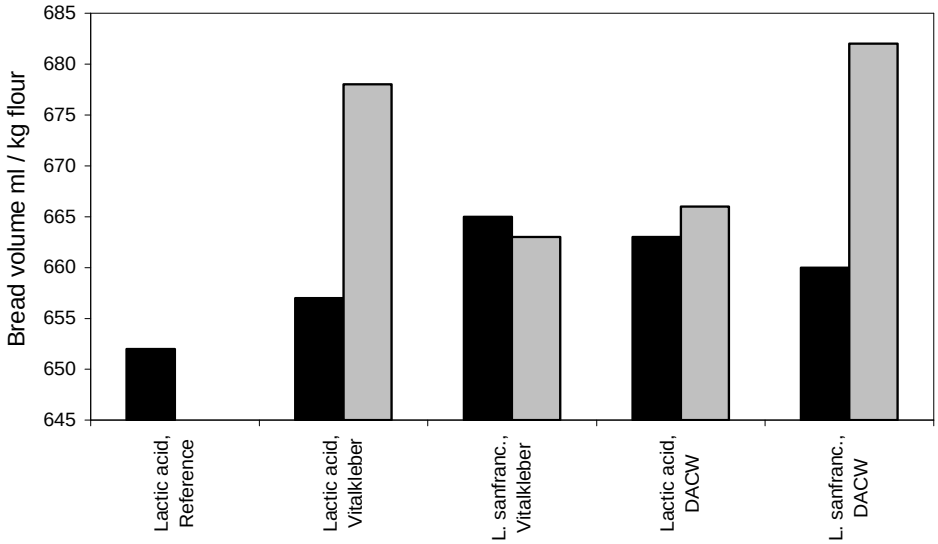

Figure 22. Influence of bakery improvers on the final bread volume. Often used products, as „Vitalkleber" and Diacetyl-tartaric-acid-ester (DATEM) were supplemented to sourdoughs fermented with *L. sanfranciscensis* TMW1.53 or to chemically acidified (with lactic acid adjusted to a pH-value of 3.6) doughs. After 20 hours of fermentation a 10% sourdough dosage was added to the final bread dough.

This approach was executed with microbiological preferments and with chemically acidified preferments. The supplemented bakery improvers were not degraded during wheat sourdough fermentations and showed a positive impact on bread volume (two outliers, black bar: lactic acid acidified sourdough with Vitalkleber, and *L. sanfranciscensis* sourdough with DATEM). Thus, their effective application in wheat sourdough fermentations is possible. In another approach, with addition of Vitalkleber and DATEM to wheat sourdoughs fermented with *L. sanfranciscensis*, the increase in final bread volume could also be seen (see table 10).

The addition of Vitalkleber and DATEM in sourdoughs fermented with *L. sanfranciscensis* caused an increase in ethanol accumulation after 20 hours, 140.8 mmol/kg dough (Vitalkleber) and 156.4 mmol/kg dough (DATEM), respectively; in comparison with ethanol values of 31.2 mmol/kg dough in sourdough fermented with *L. sanfranciscensis* without the addition of bakery improvers (data not shown). Noticeably, the high fermentation quotients of 10.8 after 5 hours, and 14.5 after 20 hours in sourdoughs fermented with *L. sanfranciscensis* supplemented with Vitalkleber. The final bread volume was increased by 1.4% in sourdoughs fermented with *L. sanfranciscensis* supplemented with Vitalkleber, and approximately 5.8% in sourdoughs fermented with *L. sanfranciscensis* supplemented with DATEM in comparison with the bread volume gained from sourdoughs fermented with *L. sanfranciscensis* without added bakery improvers.

The impact of the supplemented bakery improvers on the protein degradation during sourdough fermentations was evaluated at the Hans-Dieter-Belitz Institute for Cereal Grain Research by Dr. Wieser (see also report AiF 14492 N). In conclusion, by addition of DATEM during wheat sourdough fermentations no differences were observed in respect to protein degradation; the protein deficiency after 24 hours of fermentation was compensated by supplementation of Vitalkleber at the beginning.

Table 10. Characterization of the fermented sourdoughs supplemented with bakery improvers

Approach	**Time (h)**	**Plate count cfu/g dough**	**pH**	**Lactate**	**Acetate**	**Fermentation quotient**	**Bread volume ml/kg flour**
				mmol/kg dough			
Chemically	0	$< 10^4$	5.94	n.d.	n.d	---	---
acidified	5	$< 10^4$	5.26	14.2	6.4	2.2	---
	20	$< 10^4$	4.61	48.6	21.7	2.2	696.1
Chem. acid.	0	$< 10^4$	6.03	n.d.	n.d	---	---
+ Vitalkleber	5	$< 10^4$	5.26	10.9	3.3	3.3	---
	20	1.3×10^6	4.64	31.0	10.9	2.8	682.5
Chem. acid.	0	$< 10^4$	5.70	n.d.	n.d.	---	---
+ DATEM	5	$< 10^4$	5.10	15.8	6.2	2.5	---
	20	1.0×10^6	4.91	50.2	13.8	3.6	684.3
L. sanfrancis-	0	1.6×10^7	5.53	n.d.	n.d.	---	---
censis	5	3.0×10^7	4.95	7.1	0.9	7.9	---
	20	7.3×10^8	3.66	32.6	6.8	4.8	636.8
L. sanfrancisc.	0	1.3×10^7	5.65	n.d.	n.d.	---	---
+ Vitalkleber	5	4.2×10^7	5.06	5.4	0.5	10.8	---
	20	1.2×10^9	3.74	48.0	3.3	14.5	645.7
L. sanfrancisc.	0	1.9×10^7	5.38	n.d.	n.d.	---	---
+ DATEM	5	3.5×10^7	4.83	5.0	3.9	1.3	---
	20	7.5×10^8	3.99	46.1	9.5	4.9	673.6

2.3 DISCUSSION

2.3.1 Effect of endogenous and exogenous parameter on the final bread volume during wheat sourdough fermentations

A classification of the different parameters, e.g. sourdough dosage, fermentation strain selection in respect to final bread volume could not be established due to diverse reasons. As evidence, the results obtained in figure 7 and 12 showed no trend in consideration of percentage of sourdough dosage to the final bread dough or selected sourdough fermentation organism. In further studies, attention should be paid to the physiological status of the microorganisms used in the sourdough fermentations. Reproducibility of the sourdough fermentations could be analyzed with the determination of total plate count, pH-value, and the fermentation quotient in the dough system. Thus, sourdough fermentations with comparable values of the three mentioned parameters might enable a classification based on strain selection or sourdough dosage in respect to final bread volume. A classification should be established based on worst case scenarios; therefore, the addition of different percentages of sourdough dosage to the final bread dough should cover maybe 0%, 20%, and 40%. The sourdough dosage to the final dough in fermentations with different microorganism should be at least 20%. The recommended addition of sourdough to the final bread dough is approx. 12% for wheat breads (4). Possible differences caused by the sourdough microbiota in respect to final bread volume could be detected when increasing amounts of sourdough dosage are used. The classification must be established in independent baking approaches (days), because the physiological status of the applied yeasts varies on a daily base in the baking process. The usage of dry yeast is not recommended (see chapter 2.2.4, figure 13). Overall, with the insufficient bread volume data generated in this study no clear classification could be established; further studies have to generate more data in respect to final bread volume for the parameters sourdough dosage and the selection of fermentation microorganisms, respectively.

The accumulation of organic acids produced by the microbiota during wheat sourdough fermentations could inhibit the baker's yeast in the baking process resulting in a decreased final bread volume. The obligate heterofermentative hexose metabolism accumulates lactate, ethanol and CO_2 as the major products unless co-substrates are available that enable the regeneration of reduced cofactors. In the presence of external electron acceptors, acetyl-phosphate is converted to acetate with the yield of an additional molecule of ATP (10). Several co-factors, e.g. O_2, fructose, oxidized glutathione could be reduced by specific

enzymes (see also chapter 4 of this thesis), and therefore enable regeneration of the oxidized form of pyridine nucleotide (10, 23, 30, 31). The inhibition of the yeast by high amounts of acetic acid was shown in chapter 2.2.3; the yeast exhibited a strong decrease in ethanol accumulation in doughs supplemented with acetic acid. The inhibitory effect of organic acids is caused by the undissociated form of the acid. Therefore, due to the higher pK_a of acetic acid compared to lactic acid, the antimicrobial activity of acetate is higher at equivalent pH values and the same total concentrations of acids (1). The undissociated acetic acid diffuses freely into the yeast cell. Once inside the cell, the acid dissociates due to higher intracellular pH and causes acidification of the cytoplasma (34). Under fermentation conditions, the intracellular pH of *Saccharomyces cerevisiae* is usually maintained between 5.5-5.75 when the external pH is 3.0 (19). Thomas et al. (34) proposed that the total concentration of acetic acid determines the extent of growth inhibition by *S. cerevisiae*, not the concentration of the undissociated form of the acid alone. Figure 9 clearly demonstrates the above mentioned phenomenon, the final bread volume drastically decreased in sourdough breads supplemented with acetic acid. Fermentation arrest and reduced ethanol volumetric production of *S. cerevisiae* was shown in sourdough fermentations (this study) and during alcoholic fermentations (11, 13, 24, 28).

Endogenous ascorbic acid oxidase from flour could explain the effect of ascorbic acid on the final bread volume as shown in figure 14. The enzyme was characterized and stable within a pH-range of 5 to 9 (21); in another study from Every (9), optimal pH-values of 6.2-6.3 were estimated for the endogenous flour ascorbate oxidases. Moreover, the involved endogenous glutathione dehydrogenase in the enzyme cascade was found to have their optimal activity in a pH-range of 5.5-7.5 (22). Therefore, the impact of supplemented ascorbic acid on an increase of final bread volume is pH-dependent; enzymes involved in the cascade (figure 14) have an optimal activity above a pH-value of 5. The final bread volume prepared with sourdough (+ ascorbic acid, pH: 4.7) was even decreased in respect to the volume compared with the reference bread (see figure 15); the decreased volume is caused by the pH-drop and the resulting effect on the gluten network. A possibly inhibitory effect of ascorbic acid on the fermentation performance of the baker's yeast is unlikely due to the antioxidative effect of the compound.

2.3.2 Effect of redox reactions and glutathione reductase on thiol-exchange reactions in wheat sourdoughs.

The addition of the reduced and oxidized form of glutathione during wheat sourdough fermentations with *L. sanfranciscensis* showed no influence in respect to the final bread volume compared with chemically acidified doughs. Thus, a direct impact of the microbial glutathione reductase during the fermentation period on the gluten network resulting in decreased final bread volumes could be excluded with this approach. Although, differences in final bread volume could be compensated due to addition of fresh dough and fermentation activity of the baker's yeast, again it should be noted that only 10% of sourdough dosages were introduced into the final bread doughs. In comparison, it was concluded that in addition to the pH-dependent activity of cereal proteases, redox reactions catalyzed by lactobacilli determine gluten quality during sourdough fermentations. Thus, the formation of thiols by *L. sanfranciscensis* interferes with gluten polymerization during sourdough fermentations (37).

In contrast to the results of glutathione addition during sourdough fermentations is the outcome of the approach using a cognate mutant of *L. sanfranciscensis* decreased in glutathione reductase activity. The functional characterization of a glutathione reductase in lactobacilli was carried out using a GshR-deficient mutant of *L. sanfranciscensis* TMW1.53. Although the enzyme is located in the cytoplasm, the accumulation of thiols in the extracellular medium was attributable to GshR activity. Import of glutathione was previously demonstrated in *S. mutans* (29); however, glutathione in wheat doughs undergoes thiol-exchange reactions with cyst(e)ine and other thiols (14). Thus, transport of reduced or oxidized thiol compounds other than glutathione across the cytoplasmic membrane may account for the effects of metabolism on extracellular thiol levels. Previously, the intracellular conversion of cystine or cysteine in *L. reuteri* was shown to increase extracellular thiol levels (17, 18).

L. sanfranciscensis increased thiol levels in wheat doughs, whereas a decrease of thiols was observed in wheat doughs fermented with *L. sanfranciscensis* TMW1.53Δ*gshR*. The extracellular accumulation of thiols is particularly relevant in wheat doughs. The quality and quantity of gluten proteins in wheat flours is of paramount importance for wheat bread quality and the intermolecular disulfide crosslinks of glutenin subunits to form the glutenin macropolymer are dependent on the presence or absence of low molecular weight sulfhydryl compounds (14). The elasticity and viscosity of wheat sourdoughs decreases during fermentation because of altered protein net charge, disruption of thiol-crosslinking of gluten

protein, and proteolytic degradation of glutenin subunits (3, 8, 32, 37). The disruption of disulfide crosslinks in the gluten macropolymer occurs early during fermentation and is dependent on the presence of heterofermentative lactic acid bacteria in the dough (37). The direct impact of the glutathione reductase of *L. sanfranciscensis* on gluten proteins during sourdough fermentations was shown in figure 21. The degradation of the glutenin fraction was deviating by approx. 20% between the wild type strain of *L. sanfranciscensis* and the mutant TMW1.53Δ*gshR*. Thus, the knockout of a single enzyme activity resulted in a decreased glutenin network breakdown during wheat sourdough fermentations. In the study by Wieser et al. (40), the glutenin degradation showed comparable results in respect to *L. sanfranciscensis* and chemical acidification during sourdough fermentations, respectively. They assumed that the proteolytic activities of lactobacilli played only a marginal role in gluten degradation.

In comparison, proteolytic degradation of gluten proteins occurs only after extended fermentation times corresponding to pH values of less than 4.5 and a comparable extent of gluten proteolysis occurs in aseptic and fermented doughs (32, 33). In keeping with the different time scales of proteolysis and thiol exchange, a comparison of fundamental rheological properties of aseptic acidified wheat doughs and sourdoughs with the same pH revealed significant differences between fermented and unfermented doughs after 6 hours of fermentation but not after 24 hours of fermentation (8). Thiol exchange reactions in wheat doughs are furthermore relevant in applications targeting complete proteolytic degradation of gluten proteins. In wheat doughs, proteolysis is limited by the activity of proteolytic enzymes but the substrate solubility becomes the limiting factor to protein degradation upon addition of external proteases (12, 32, 36). The disruption of disulfide crosslinks between gluten proteins by chemical reducing agents or heterofermentative lactobacilli is required to achieve a virtually quantitative hydrolytic degradation of gluten proteins in wheat doughs (32, 36).

The effects of *L. reuteri* and *L. pontis* on thiol levels in sourdough were comparable to *L. sanfranciscensis* but these strains did not exhibit glutathione reductase activity. Remarkably, *L. reuteri* BR11Δ*cyuC*, which has a comparable phenotype as the *gshR* deficient *L. sanfranciscensis* with respect to its tolerance to oxygen (see chapter 4 and (35)), increased the thiol levels in dough when compared to the cognate wild type strain, indicating a role of cystine metabolism via cystathionine-γ-lyase for thiol-exchange reactions in wheat doughs. The effect of CyuC deletion in *L. reuteri* was less pronounced compared to the GshR deletion in *L. sanfranciscensis*. It is counter-intuitive that the loss of cystine transport increased

extracellular thiol levels; however, the *L. reuteri* CyuC mutant strain remains capable of extracellular accumulation of thiols from substrates other than cystine (18, 25).

Bakery improvers like Vitalkleber and emulsifiers, e.g. diacetyl-tartaric-acid ester (DATEM) are widely used in bakeries as dough strengtheners, and finally increase the bread volume. With the approaches conducted in this study, the supplementation of the improvers during sourdough fermentations was evaluated in respect to their inactivation/degradation by the fermentation microbiota resulting in a decreased or not enhanced final bread volume. No differences in respect to protein degradation were observed by the addition of the emulsifier DATEM in sourdough fermentations; the protein deficiency after 24 hours of fermentation was compensated by supplementation of Vitalkleber at the beginning. The bread volumes achieved with a 10% dosage of sourdough to the final bread dough were increased by the usage of both bakery improvers. Therefore, the possibility of bakery improver supplementation during wheat sourdough fermentations was shown in consideration of enhanced bread volumes. Aroma and taste analyses should be performed due to increased values for ethanol in sourdoughs fermented with *L. sanfranciscensis* and supplemented Vitalkleber or DATEM, respectively. These analyses should focus also on the shifted fermentation quotient in favor to lactic acid in sourdoughs fermented with *L. sanfranciscensis* by the addition of Vitalkleber.

Future perspectives should focus not only on single enzyme reactions by the sourdough microbiota, but attention should be paid on an overall screening approach of different lactobacilli in regard to redox potential during wheat sourdough fermentations. The redox potential discriminates among species of lactic acid bacteria during fermentations (5). Thus, an overall perception concerning the reductive or oxidative potential of lactobacilli during fermentations could lead to the best selection of fermentation organism in respect to the desired quality parameter.

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3

3. DISTRIBUTION OF ENZYME ACTIVITIES IN LACTOBACILLI ABLE TO GENERATE VOLATILE AROMA COMPOUNDS FROM HYDROXYCINNAMIC ACIDS

Pentosans are the prominent non-starch polysaccharides in respect to the bread production process (see also chapter 1). The solubility of pentosans (WE-AX and WU-AX) is influenced by the composition and length of side-chains, the degree of substitution, and the predominant linkages (1); thus, they could be modified due to degradation caused by endogenous enzymes or by the activity of sourdough associated microorganisms during fermentation. Those enzyme activities could account for the degradation of the arabinoxylan backbone during wheat sourdough fermentations liberating free ferulic acid.

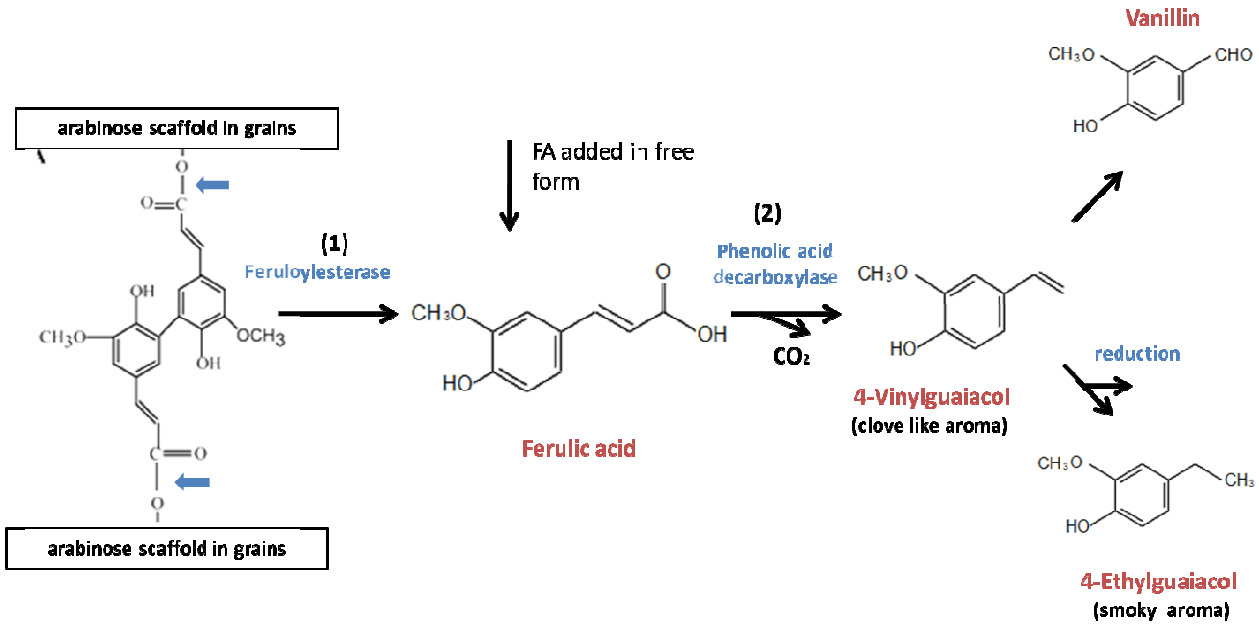

Figure 23. Schematic presentation of enzymes (blue colored) involved in ferulic acid (FA) release and conversion to volatile aroma compounds (red colored). (1) Feruloylesterase, (2) Phenolic acid decarboxylase (Pdc/Pda) (Vogel et al., unpublished).

The optimal pH-range of 4-5 for endogenous arabinoxylan degrading enzymes (e.g. α-L-arabinofuranosidase, β-D-xylosidase, and β-1,4-xylanase) is reached expeditious during

sourdough fermentations. In chemically acidified rye sourdough fermentations a decrease of the total arabinoxylan content resulted in an increase of WE-AX based on acid hydrolysis (6).
Feruloylesterases [E.C. 3.1.1.73] are a subclass of carboxylic acid esterases [E.C. 3.1.1.1] that are able to hydrolyze the ester bond between hydroxycinnamic acids and sugars present in the plant cell walls (51). It is common for esterases to be active on a broad range of substrates. The use of small, soluble substrates allows the determination of kinetic constants, giving some information on the affinity and catalytic efficiency (51). Although these enzymes exhibit different substrate specificities, which may overlap with those of other esterases (e.g. acetyl xylanesterases, general esterases), they are all specific for the hydrolysis of hydroxycinnamoyl esters, which is evident from their K_m and specific activity values for hydroxycinnamate esters relative to that for general substrates such as *p*-nitrophenol acetate (30). Feruloyl esterases have been purified, overexpressed and characterized from a wide variety of microorganisms, e.g. *A. brasiliensis* and *A. tubingensis* (18), containing the GXSXG motif, a common conserved sequence in lipases (GHSLG). An extracellular feruloyl esterase has been isolated and purified from the thermophilic *Clostridium stercorarium* grown on birchwood xylan (20), the psychrophilic bacterium *Pseudoalteromonas halosplanktis* TAC125 possesses a gene coding for a cold-adapted feruloyl esterase (3). The genes encoding CinA and CinB from the ruminal bacterium *Butyrivibrio fibrisolvens* E14 have been expressed at high levels (15, 16), also a novel recombinant ethyl ferulate esterase from *Burkholderia multivorans* has been characterized (45).
Liberated ferulic acid could be further decarboxylated (see also Figure 23) either by microbial enzyme activities (Pdc/Pda) or during thermal release in the beer production process (11, 50). The resulting capable phenolic aroma precursors, e.g. 4-vinyl-2-methoxyphenol are undesirable in the beer or wine production process, but could lead to eligible aroma components in wheat sourdough fermentations. In earlier fermentation studies with lactobacilli in nutrient broth supplemented with ferulic acid it was elucidated, that specific strains liberated clove- and vanilla-like flavors. Hydroxycinnamic acids, e.g. ferulic acid, caffeic acid, and coumaric acid could be metabolized with the help of lactobacilli during wheat sourdough fermentations to odor active substances. Besides, 4-vinyl-2-methoxyphenol (clove-like aroma) originated from decarboxylation of ferulic acid, also 4-ethyl-2-methoxy-phenol (smoky aroma), vanillin (vanilla aroma), and 2-methoxyphenol (smoky aroma) could be generated during reduction-, oxidation- and decarboxylation reactions. The production of vanillin from simple phenols by wine-associated lactic acid bacteria was determined by

Bloem et al. (5). The ferulic acid metabolism via decarboxylation to produce 4-vinylguaiacol and a further conversion to vanillin as proposed in figure 23 was demonstrated by Li et al. (34).

In previous studies, the release of ferulic acid during wheat sourdough fermentations with lactobacilli was shown by Wieser et al. (unpublished). These measurements were performed in addition to the analyses mentioned in chapter 2.

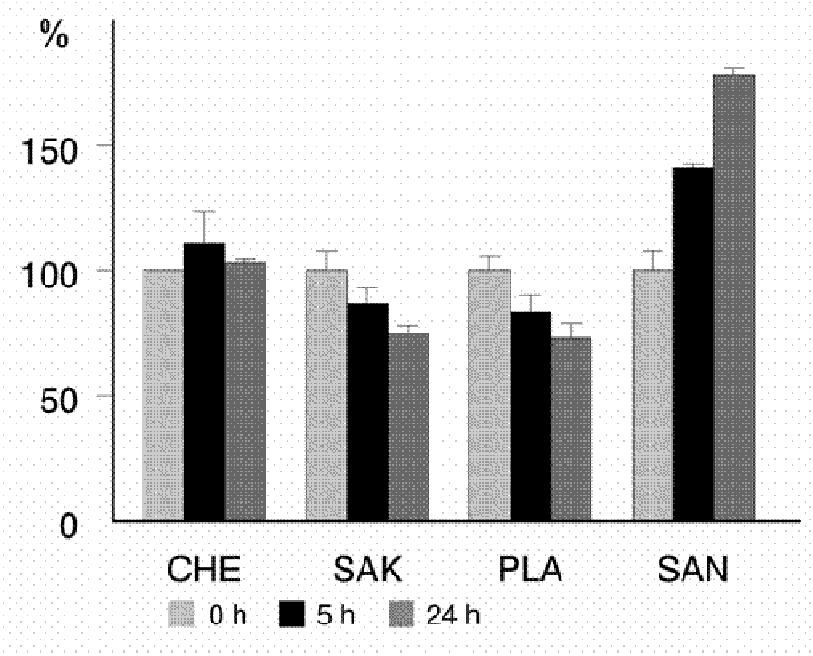

Figure 24. Content of free ferulic acid during wheat sourdough fermentations with lactobacilli. CHE, chemically acidified dough; SAK, *L. sakei* sourdough; PLA, *L. plantarum* sourdough; SAN, *L. sanfranciscensis* sourdough.

The total free ferulic acid content was 2.36 ± 0.05 µg/g dry mass in the flour „Tommi“, and following initial ferulic acid concentrations of the doughs were determined for the different approaches: chemically acidified dough 2.55 ± 0.01 µg/g; *L. plantarum* dough 2.76 ± 0.16 µg/g; *L. sakei* dough 3.19 ± 0.25 µg/g; and *L. sanfranciscensis* dough 3.13 ± 0.26 µg/g. The ferulic acid content from sourdoughs fermented with *Lactobacillus sanfranciscensis* increased during a 24 hour period, whereas the free ferulic acid content decreased in the two other fermented doughs with *L. sakei* and *L. plantarum* over the fermentation period; the content of ferulic acid remained constant in the chemically acidified control. This above-mentioned result was the starting point for the evaluation of lactobacilli able to release ferulic acid during wheat sourdough fermentations and further to generate volatile aroma compounds.

By using database analysis the sequenced *Lactobacillus* genomes were examined for putative arabinoxylan degrading enzymes to identify distinctive features for the metabolism of plant cell wall backbone in lactobacilli. The genome of *L. brevis* ATCC 367 (TMW1.1326) is

harboring two genes for α-L-arabinofuranosidases, and two β-xylosidase genes (see also appendix, chapter 5). The present study showed the heterologous cloning and biochemical characterization of a α-L-arabinofuranosidase and β-xylosidase, respectively from *Lactobacillus brevis* TMW1.1326. Moreover, the distribution of esterase activity in lactobacilli was shown able to hydrolyze phenolic acid esters. The physiological tests for predicted cinnamoyl esterase activity could be ensured on a genetically background. Predicted esterases of lactobacilli able to hydrolyze esterifies hydroxycinnamates were heterologously expressed and characterized. Moreover, the fate of the free liberated ferulic acid was verified by a PCR-screening for phenolic acid decarboxylase genes in selected lactobacilli. In fermentation studies with lactobacilli using mMRS broth supplemented with ferulic acid, coumaric acid or wort, the fermentation supernatants were analysed by HPLC to determine the degradation of the substrates to capable aroma compounds, e.g. 4-vinyl-2-methoxyphenol.

3.1 MATERIALS AND METHODS

3.1.1 Used strains, primer, media, and restriction enzymes

3.1.1.1 Used strains

Table 11. Used microorganisms and their cultivation conditions

Strain	TMW	Cultivation conditions	Source, Reference
B. subtilis	2.46	LB, 30°C, aerobic	DSM 347
W. confusa	1.928	MRS, 30°C, anaerobic	ACA-DC 3396
L. amylovorus	1.653	MRS, 37°C, anaerobic	SER Sauerteig 1/5
L. amylolyticus	1.1078	MRS, 48°C, anaerobic	Hofbräuhaus FS
L. zymae	1.943	MRS, 30°C, anaerobic	ACA-DC 3411
L. hilgardii	1.1298	MRS, 37°C, anaerobic	BöckerKr-E/2
L. brevis	1.1326	MRS, 30°C, anaerobic	ATCC 367
L. pentosus	1.10	MRS, 30°C, anaerobic	DSM 20314^T
L. perolens	1.501	MRS, 30°C, anaerobic	Gent LAB 1191
L. plantarum	1.460	MRS, 30°C, anaerobic	Gent LAB 1146
L. plantarum	1.468	MRS, 30°C, anaerobic	Gent LAB 1158
L. spicheri	1.262	MRS, 30°C, anaerobic	DSM 15429^T
L. sanfranciscensis	1.53	MRS, 30°C, anaerobic	DSM 20451^T
L. sanfranciscensis	1.1304	MRS, 30°C, anaerobic	BRS1
L. fructivorans	1.1253	MRS, 30°C, anaerobic	DSM 20203^T
L. farciminis	1.68	MRS, 30°C, anaerobic	DSM 20184^T
L. mindensis	1.1206	MRS, 30°C, anaerobic	DSM 14500^T
L. rossiae	1.164	MRS, 30°C, anaerobic	Bier 23, Dr. Vogel
L. sakei	1.22	MRS, 30°C, anaerobic	LTH 677
L. paralimentarius	1.256	MRS, 30°C, anaerobic	DSM 13238
L. alimentarius	1.62	MRS, 30°C, anaerobic	DSM 20249^T
L. casei	1.1250	MRS, 30°C, anaerobic	DSM 20001^T
L. nantensis	1.1265	MRS, 30°C, anaerobic	GO-S11
L. helveticus	1.1176	MRS, 37°C, anaerobic	DSM 20075^T
L. delbrueckii	1.1379	MRS, 37°C, anaerobic	CLbDb 79
L. johnsonii	1.1179	MRS, 37°C, anaerobic	DSM 10533^T
L. crispatus	1.143	MRS, 37°C, anaerobic	Gerti (pig faeces)
L. fermentum	1.890	MRS, 37°C, anaerobic	WVS 48/B4
L. acidophilus	1.697	MRS, 37°C, anaerobic	Nestlé, CRN La1
L. gasseri	1.1173	MRS, 37°C, anaerobic	DSM 20243^T
L. pontis	1.1086	MRS, 37°C, anaerobic	DSM 8475^T
L. pontis	1.1463	MRS, 37°C, anaerobic	LTH 4819
L. panis	1.1318	MRS, 37°C, anaerobic	Wort isolate
L. frumenti	1.103	MRS, 37°C, anaerobic	Isolate M. Müller
L. reuteri	1.106	MRS, 37°C, anaerobic	Isolate M. Müller
L. buchneri	1.1162	MRS, 37°C, anaerobic	DSM 20057^T
Bifidobact. lactis	2.462	BIFIDO, 37°C, anaerobic	DSM 10140^T
E. coli TOP10	2.580	LB, 37°C, aerobic	Invitrogen TOP10

All lactobacilli strains used in this study are aerotolerant and were incubated under anaerobic conditions. Broth or agar plates of mMRS were used for cultivation of the strains at the stated temperatures (see table 11). Dilution streaks were performed on agar plates and incubated for 24 hours to ensure the purity of the cultivated strains. Using the pure dilution streak, a pre culture of the specific strain in broth was inoculated, and a second culture using 1% of the first culture was inoculated in 10 ml mMRS. The working culture was then inoculated from the second culture and incubated overnight. Broth cultures were incubated in sterile, gas-tight Sarstedt-test tubes (normally 50 ml). Agar plates were incubated in an anaerobic chamber using 80% of nitrogen and 20% carbon dioxide gas. *E. coli* strains were incubated aerobically in Erlenmeyer flasks on a horizontal shaker (220 U/min) in LB-broth at 37°C, for maintaining plasmids 100 μg x L^{-1} ampicillin was added.

3.1.1.2 Primers

Table 12. Used primers for PCR and cloning experiments

Primer	**Sequence (5` to 3`)**	**Tm [°C]**
GASS-Klon-for	TAT ACC ATG GTT ATG AAG TTA AAG AAA AAG	63.0
GASS-Klon-rev	TAT ATC TAG ACG AAA AGT ATT ATT ATC TTG	61.0
GASS-for1	CTT AAA CTT GGTT GGA AC	49.0
REU-Klon-for	TAT ACC ATG GAA ATA ACA ATC AAA CG	60.0
REU-Klon-rev	TAT ATC TAG ACG ATT TTT TAA AAA GTT AGC TAC	64.0
REU-for1	CGA CGA TTA AAA ACG ATA C	47.0
PLA-Klon-for	TAT ACC ATG GTT ATG ACA TCG ATG GAA TTT AAG	68.0
PLA-Klon-rev	TAT ATC TAG AAA TTT AAA CGC GGC CAG TGC TAA	69.0
PLA-for1	TTC TGC GTT TTG ACT TCA	49.0
1890-Klon-for	TAT ACC ATG GAA GTT GCA ATC AAG A	61.0
1890-Klon-rev	TAT ATC TAG ATT GTT TAA GAA ATC GGC CAC	65.0
1890-Est-for1	GTT TTA AGG GTG ACC TAG	51.0
HELV-Klon-for	TAT ACC ATG GTT ATG TCC CGC ATT ACG ATT G	70.0
HELV-Klon-rev	TAT ATC TAG ATT AAA CGC AGG TTT TAA AAA TTG C	65.0
HELV-for1	GTT CGT TTT GAT TTT AAT G	46.0
ACID-Klon-for	TAT ACC ATG GTT ATG TCT CGC ATTA ACA ATT G	67.0
ACID-Klon-rev	TAT ATC TAG ACG AAA TAG GGG CTT CAA AAA TTC	68.0
ACID-for1	CAA ATT AGC CGA TAA CTT A	49.0
pBAD-for	CTA CTG TTT CTC CAT ACC CG	58.0
pBAD-rev	CTG ATT TAA TCT GTA TCA	44.6
AFN I	CCN GGN GGN AAY TTY GT	48.0-58.0
AFN II	GGN AAY GAR ATG GAY GG	48.0-56.0
AFC	CCA NAC RTT CCA YTC RTC	50.0-58.0
arabino-2-for	CAT TAA AAC GTG GTG TTT G	50.2
arabino-2-Klon-for	TAT ACT CGA GAT GCA AGG TTC TAC AAA G	62.2
arabino-2-Klon-rev	TAT ATC TAG ATT ATC TCG GAG CTT AAA CCG	64.0
b-xyl-1-for	CAT TTT GAA GGA TTT GAT C	48.0
b-xyl-1-Klon-for	TAT ACC ATG GTT ATG ACT TTA ATT CAA AAT	58.0

b-xyl-1-Klon-rev	TAT ATC TAG AAA TTC ATG AGT TAA GTC CTC	60.0
pdc-f	GAN AAY GGN TGG GAR TAY GA	(17)
pdc-r	GGR TAN GTN GCR TAY TTY T	50°C
PAD-for	CCA CAC CGT TGA TTA CCG	56.0
PAD-rev	GAT AAG TGG CAT ACT TTT CAC G	56.5
coumaric-for	GGC ACA CAC TTT ATY TAC AC	54.2
coumaric-rev	TTG RTA AGT GAC CGT AAT TTC	53.0

3.1.1.3 Media and buffers

All the common used media and buffers are listed in the appendix at the end of this thesis (see chapter 5). Following, specific buffer solutions were used for the determination of the pH-dependent cinnamoyl esterase-activity using spectrophotometer. The evaluation of the heterologously in *E. coli* TOP10 expressed cinnamoyl esterase-activity of predicted cinnamoyl esterases, after purification using FPLC was performed with the help of a spectrophotometer (Tecan) measured in a pH-range of 4.0–9.0. The pH-values were gradually increased with 0.5 steps up to a pH-value of 9.0. For the different pH-ranges the indicated buffers (see tables 13-16) were used.

Table 13. Used buffer solutions for the pH-dependent determination of cinnamoyl esterase-activity

pH-value	**buffer**
4.0-6.0	Citrate buffer
6.5-8.0	Sodium phosphate buffer
8.5-9.0	Tris-HCL-buffer

The preparation of citrate buffer was performed, as follows: 21.01 g of citric acid was dissolved in distilled water, mixed with 200 ml of sodium hydroxide (1 M), and filled up to 1000 ml with distilled water. 100 ml buffer solution with the respective pH-value was prepared as shown in table 14; the citrate buffer solution – hydrochloric acid (1 M) up to pH-value of 4.5 and citrate buffer solution - sodium hydroxide (1 M) up to pH-value of 6.0 were mixed in the appropriate relation according to table 14. The final pH-values of the solutions were controlled using a pH meter.

Table 14. Preparation of citrate buffer solution in the pH-range of 4.0–6.0

pH	**Citrate solution in ml**	**Hydrochloric acid in ml**	**Sodium hydroxide in ml**
4.0	56.0	44.0	---
4.5	71.9	28.1	---
5.0	96.4	---	3.6
5.5	72.3	---	27.7
6.0	59.6	---	40.4

The preparation of sodium phosphate buffer was performed using a mixture of di-sodium hydrogen phosphate-di-hydrate (Na_2HPO_4 x 2 H_2O) and sodium di-hydrogen phosphate hydrate solutions (1 M). 177.99 g Na_2HPO_4 x 2 H_2O and 119.98 g NaH_2PO_4 x H_2O were dissolved in 1000 ml distilled water. 100 ml buffer solution with the respective pH-value was prepared as shown in table 15; the final pH-values of the solutions were controlled using a pH meter.

Table 15. Preparation of sodium phosphate buffer solution in the pH-range of 6.5–8.0

pH	Na_2HPO_4 x 2 H_2O in ml	NaH_2PO_4 x H_2O in ml
6.5	18.2	81.8
7.0	57.7	42.3
7.5	66.1	33.9
8.0	93.2	6.8

The preparation of Tris (hydroxyl methyl)-amino methane – hydrochloric acid buffers (Tris-HCL) was performed, as follows: 121.14 g Tris-HCL was dissolved in 1000 ml distilled water. 100 ml buffer solution with the respective pH-value was prepared as shown in table 16; 50 ml Tris-HCL solution was mixed with hydrochloric acid (1 M) and distilled water. The final pH-values of the solutions were controlled using a pH meter.

Table 16. Preparation of Tris-HCL buffer solution in the pH-range of 8.5–9.0

pH	Tris-HCL in ml	Hydrochloric acid in ml	Distilled water in ml
8.5	50.0	14.7	35.3
9.0	50.0	5.7	44.3

The prepared buffer solutions were stored at 4°C; a defined amount of substrate was added to the buffer solutions to evaluate the predicted cinnamoyl esterase activity.

3.1.1.4 Plasmids and general molecular techniques

The plasmid pBADB/*Myc*-His B (Invitrogen) was used for all cloning approaches in *E. coli* TOP10 (see also chapter 5.1.6). General techniques regarding cloning, DNA manipulations, and agarose gel electrophoresis were performed as described by Sambrook et al. (48) Chromosomal DNA of *Lactobacillus* strains were isolated according to the method of Lewingston et al. (33), and *E. coli* plasmid DNA was isolated with the PegGold plasmid miniprep kit I from Peqlab GmbH (Erlangen, Germany). Restriction endonuclease digestions and ligations with T4-DNA ligase were performed following the recommendations of the supplier (Fermentas, St. Leon-Rot, Germany). PCR was carried out in thermocyclers (Primus 96^{plus}, MWG-Biotech, Ebersberg, Germany) by using Arrow-Taq Polymerase and dNTP's

from MP Biomedicals (Heidelberg, Germany). PCR-products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing of PCR products or plasmids was performed by GATC. Transformations were performed with a Bio-Rad gene pulser apparatus (Bio-Rad Laboratories, USA) in 0.2 mm cuvettes (Biozym Scientific GmbH, Germany) at 2.5 kV, 25 µF and 200 Ω for *E. coli*. Electro competent *E. coli* cells were prepared according to chapter 5.1.8.
Following restriction endonucleases were used in this study: *Nco* I, *Xho* I, *Xba* I (Fermentas).

3.1.2 Preparation of mMRS-media with supplemented ethyl ferulate (EF)

For cultivation of lactobacilli mMRS-media was prepared according to chapter 5.1.7. Ethyl ferulate was dissolved in DMSO and sterile filtered; ethyl ferulate (final concentration of 0.1%) was added to 50 ml mMRS broth and inoculated with 1 ml overnight grown *Lactobacillus* suspension for fermentation studies.

3.1.3 Predicted cinnamoyl esterase activity of lactobacilli determined with plate screening assay

Cinnamoyl esterase activity in lactobacilli was evaluated using mMRS agar plates supplemented with 0.1% ethyl ferulate. Solidified agar plates showed a distinct turbidity due to existence of ethyl ferulate. The screening assay was performed with sterile sensi-discs (BD Diagnostics, Germany) wetted with 20 µl of an overnight grown culture of the specific *Lactobacillus* strain, placed in the middle of an agar-plate. The prepared plates were incubated for three days under anaerobic conditions at the appropriate temperatures. The predicted cinnamoyl esterase activity was measured as the diameter of a clearing zone around the point of inoculation expressed in millimeters. LB agar-plates supplemented with 0.1% ethyl ferulate, 75 µg x L^{-1} ampicillin for maintaining plasmids, and a final concentration of 2 mM arabiniose were used for the determination of recombinant cinnamoyl esterase activity in *E. coli* TOP10.

3.1.4 Fermentations with different lactobacilli in broth supplemented with cinnamic acid derivatives

The existence of cinnamoyl esterase activity in lactobacilli was evaluated in fermentation approaches using different cinnamic acid derivatives, e.g. ethyl ferulate, methyl coumarate, and methyl caffeate, respectively. Fermentations were performed in 50 ml Erlenmeyer flasks with mMRS broth inoculated with 1 ml of an overnight grown pre culture. The incubation of

the flasks was performed on a horizontal shaking device (140-150 U/min) to ensure a steady mixture of the sample. The final concentration of methyl caffeate, methyl coumarate, and ethyl ferulate was 2 mM. The substances were dissolved in DMSO, sterile filtered and then supplemented to the broth.

3.1.4.1 Extraction of the phenolic end-products after fermentation

After three days of fermentation the samples were centrifuged at 7.000 U/min and 4°C for ten minutes. The supernatant was poured off, and extracted with methyl tert-butyl ether (MTBE) under vigorous shaking in a ratio of 1:1. The upper phase was removed after separation and collected in an Erlenmeyer flask; the extraction process was conducted twice to achieve a higher yield of phenolic compounds. Subsequently, the samples were poured through a folded filter and carried over in a round bottom flask. The flask was attached to a rotary evaporator; the samples were evaporated under rotation in a water bath at 45°C and negative pressure of 300-400 mbar. Finally, the concentrated samples were flushed with nitrogen till complete dryness, and then resuspended in 1.5 ml methanol.

3.1.4.2 Thin layer chromatography for the determination of phenolic compounds

The degradation of ethyl ferulate, methyl caffeate, and methyl coumarate by the selected lactobacilli during fermentation was visualized using thin layer chromatography (TLC). Standard solutions (final concentration of 10 mM) were prepared of the expected end-products, e.g. ferulic acid, coumaric acid, and caffeic acid, respectively. The TLC analyses were performed on silica gel plates 60 WF254S (Merck), 10 µl of standard solutions and 10 µl of sample solutions were applied to the plates. The mobile phase consisted of chloroform, methanol, and formic acid in a ratio of 85:15:1 (v/v/v), the TLC chamber was saturated with the mobile phase at least one hour before analysis. After separation and developing, plates were dried in the air under a safety cabinet. Phenolic compounds were visualized under ultraviolet light (254 nm).

3.1.5 Purification of heterologously expressed protein using FPLC

700 ml LB broth supplemented with 100 µg x L^{-1} ampicillin in an Erlenmeyer flask was inoculated 1% with overnight grown pre culture and incubated on a horizontal shaking device at 220 U/min and 37°C up to an optical density of 0.6-0.7 for the heterologous expression of the recombinant proteins. The *E. coli* TOP10 culture was shaken after supplementation of

arabinose (final concentration of 1 mM) for 4 hours at 30°C. The cultures were centrifuged (6000 rpm, 10 minutes, 4°C), and the cell pellet was resuspended in 50 ml buffer A (application buffer, 20 mM NaH_2PO_4, 500 mM NaCl, 50 mM imidazole, and pH-value of 7.4). Subsequently, one washing step with buffer A was performed, and the cell pellet was then resuspended in 10 ml buffer A, all washing steps were performed at 4°C with cooled buffer A. The cells were solubilized with a Sonoplus Homogeniser HD2070 (Bandelin, Bering) to liberate crude cellular extracts (cycle 0.5/90%/20 s); cell suspensions on ice were treated five-times as stated with breaks lasting at least two minutes. Finally, the crude cellular extracts were centrifuged for 15 minutes at 4°C and 12.000 U/min to separate the heterologously expressed proteins from cell debris. The purifications of the recombinant proteins were performed with FPLC (Fast Protein Liquid Chromatography) and the usage of HisTrap HP-affinity columns (see also manufacturer's instructions, Amersham Biosciences). 5 ml of the crude cellular extract was applied to the 5 ml loop of the FPLC, and a computerized concentration gradient between buffer A and buffer B (elution buffer, 20 mM NaH_2PO_4, 500 mM NaCl, 500 mM imidazole, and pH-value of 7.4) performed the purification run. While target protein was bound to the column due to the His-tag, residual crude proteins were flushed out in a washing step of five minutes (2 ml/min) with buffer A. A gradient program was performed from 100% buffer A and 0% buffer B to 0% buffer A to 100% buffer B in ten minutes (2 ml/min). Release of the target protein was achieved from the His-Trap column by running the program for five minutes (2 ml/min) with 100% buffer B. Protein-rich fractions were determined based on peak identification in the elution diagram; these fractions were immediately kept on ice till further analyses were conducted. The eluate was dialyzed (VISKING®, dialysis tubing 20/32) against 0.1 M sodium phosphate buffer (pH 7.0) at 4 °C. After 4 hours the buffer was exchanged and the dialyses was performed overnight. Determination of protein concentrations (7) and SDS-PAGE was performed according to chapter 5.1.9.

3.1.6 Determination of predicted cinnamoyl esterase activity of the purified recombinant proteins using spectrophotometer

The determination of activity according to Michaelis-Menten was performed with the substrate p-nitrophenyl acetate (p-NPA) by using a spectrophotometer (Tecan). P-nitrophenyl acetate is an achromatic, crystalline substance; p-nitrophenyl and acetate is generated after hydrolysis of p-NPA. In aqueous solutions a yellow color occurs, the absorption optimum of

p-nitrophenyl is 400 nm and the solubility in water is 16 g/l at 25°C. The substrate was often used for cinnamoyl esterase activity determinations, because of the sterical similarity to ferulic acid (22, 31, 45). The activity determination of the purified heterologous expressed proteins was performed in 100 mM sodium phosphate buffer with a pH-value of 7.0 at ambient temperature. Serial dilutions were prepared of the substrate ranging from 0.075 mM to 4.0 mM. 2 µl of sample was pipetted to 200 µl of substrate-sodium phosphate buffer. The liberated p-nitrophenyl was measured at 405 nm using a spectrophotometer; sodium phosphate buffer without supplemented substrate served as a reference. The activity of the predicted cinnamoyl esterases against the substrate p-NPA was measured in 50 cycles every 20 seconds at 405 nm. A calibration curve up to 5 mM was erected out of a serial dilution of p-nitrophenyl. K_m und V_{max}-values were evaluated after the concentration determination of liberated p-nitrophenyl using SigmaPlot 10.0. All analyses were performed in triplicate.

3.1.6.1 Determination of pH-dependent esterase acitivity

Evaluation of pH-dependent esterase activity was determined with the indicated buffer systems according to chapter 3.1.1.3. A pH-range of 4.0-9.0 was covered by using the different buffer systems, e.g. citrate buffer, sodium phosphate buffer, and Tris-HCL buffer, respectively. The model substrate p-nitrophenyl acetate was used to perform the pH-dependent activity tests. The samples were prepared as mentioned under chapter 3.1.6; all analyses were measured over 50 cycles every 20 seconds at 405 nm in triplicate.

3.1.6.2 Determination of substrate side chain length-dependent esterase activity

Evaluation of side chain-dependent esterase activity of the heterologous expressed predicted cinnamoyl esterases was determined using p-nitrophenyl acetate (p-NP-C2), p-nitrophenyl butyrate (p-NP-C4), and p-nitrophenyl caprylate (p-NP-C8) as a substrate. All measurements were performed in sodium phosphate buffer (pH-value of 7.0) at ambient temperature. The samples were prepared as mentioned under chapter 3.1.6; all analyses were measured over 50 cycles every 20 seconds at 405 nm in triplicate.

3.1.6.3 Determination of temperature-dependent esterase activity by using HPLC

The evaluation of the temperature-dependent activity of the heterologous expressed predicted cinnamoyl esterases was performed in a temperature range of 20°C, 30°C, 35°C, 40°C, 45°C, 50°C, and 60°C. Following recombinant esterases of lactobacilli were used for the

temperature-dependent characterization: *L. reuteri* TMW1.106, *L. gasseri* TMW1.1173, *L. acidophilus* TMW1.697, and *L. plantarum* TMW1.460. Ethyl ferulate as the substrate was mixed in a reaction approach with the FPLC-purified esterases. For evaluation purposes of the chromatograms different standard solutions of ethyl ferulate and ferulic acid were compared with retention times resulted from reactions with the purified esterases and ethyl ferulate as a substrate. The reaction mixture was prepared as follows: 900 µl sodium phosphate buffer (pH-value of 7.0), and 100 µl 100 mM ethyl ferulate in methanol (final concentration 10 mM) were mixed, and the enzyme kinetic reaction was started after the addition of 10 µl purified, dialyzed protein fraction. Before, the amount of added protein was determined according to Bradford (8); therefore, all approaches were performed with a standardized protein concentration. The reactions were stopped by using a heating block thermostat at 95°C. Empty 1.5 ml reaction tubes were incubated at 95°C, 500 µl reaction mixtures were pipetted into the empty reaction tubes followed by the addition of 500 µl methanol at the end of the reaction time. The reaction inactivation was continued for 2-3 minutes at 95°C. The inactivated mixture was transferred to HPLC vials by using a sterile syringe and filtration through a 0.2 µl filter. HPLC analyses were performed according to chapter 3.1.9.1.

3.1.7 Determination of α-L-arabinofuranosidase and xylosidase activity of the purified recombinant proteins using spectrophotometer

The evaluation of the temperature and pH-dependent activity of the purified, heterologous expressed enzymes was performed with substrate solutions of para-nitrophenyl arabinofuranoside (p-NP-ara), and para-nitrophenyl xylopyranoside (p-NP-xyl) in sodium phosphate buffer, respectively. 20 µl of sample was pipetted to 200 µl of substrate-sodium phosphate buffer (final concentration of substrate 2 mM). The liberated p-nitrophenyl was measured at 405 nm using a spectrophotometer; sodium phosphate buffer without supplemented substrate served as a reference. Determination of the temperature-dependent activity up to 40°C was performed directly in the spectrophotometer; higher temperatures were not supported by the apparatus. For temperature-dependent activity characterization above 40°C, the reactions were performed in 1.5 ml test tubes and the usage of a water bath. The liberated p-nitrophenyl was measured also in the spectrophotometer. The pH-dependent activity was evaluated according to chapter 3.1.1.3 and chapter 3.1.6.1, respectively. Kinetic parameters of the purified enzymes were determined by the addition of increasing concentrations from 0.5 mM to 4.5 mM of p-NP-ara and p-NP-xyl; the enzymatic activity [mmol/l] was measured in 20 cycles

every 10 seconds at 405 nm. K_m und V_{max}-values were evaluated after the concentration determination of liberated p-nitrophenyl using SigmaPlot 10.0. All analyses were performed in triplicate.

3.1.7.1 Thin layer chromatography for the determination of pentose monosaccharides

The degradation of arabinan, xylan, and wheat arabinoxylan by the recombinant enzymes from *L. brevis* TMW1.1326 was visualized using thin layer chromatography (TLC). Standard solutions (final concentration of 10 mM) were prepared of the expected end-products, e.g. arabinose, and xylose, respectively. The TLC analyses were performed on silica gel plates 60 WF254S (Merck), 10 µl of standard solutions and 10 µl of sample solutions were applied to the plates. The recombinant enzymes ara-2 and xyl-1 were added to sodium phosphate buffer (0.1 M) supplemented with arabinan, xylan, or wheat arabinoxylan (final concentration 10 mM). The reactions were stopped after 6 hours by using a heating block thermostat at 95°C. The mobile phase consisted of ethyl acetate, acetic acid, and water in a ratio of 2:1:1 (v/v/v), the TLC chamber was saturated with the mobile phase at least one hour before analysis. After separation and developing, plates were dried in the air under a safety cabinet. Sugars were visualized after spraying the TLC plates with 5% sulphuric acid in ethanol (v/v), followed by heating in a dry oven for 10 minutes at 120°C.

3.1.8 Sequencing of the cloned genes into the expression vector pBAD-*Myc*His B

Extraction of the pBAD-Myc His B vector hosting the cloned genes was achieved with peqGOLD plasmid miniprep Kit 1 according to manufacturer's instruction. The evaluation of plasmid-DNA amount in the samples was determined by using Nanodrop (peqLAB Biotechnology GmbH). The sequencing of the purified vectors was conducted by GATC Biotech AG (Konstanz, Germany) with the help of primer pBAD-for. The results of the DNA-sequencing were translated into amino acid sequences by using Transeq (EMBL) and alignment was achieved with known sequences for hydrolases/esterases in the databases with the program ClustalW (EMBL).

3.1.9 Screening of phenolic acid decarboxylase genes using PCR and verification of decarboxylase activity in lactobacilli

Genomic DNA of lactobacilli was used as a template in PCR with primers according to table 12. In previous studies, PCR was performed with primers pdc-f and pdc-r according to de las Rivas (17). All PCR´s were performed as stated in annex chapter 5, with the specific

annealing temperatures of the respective primers. The activities of decarboxylases were determined in fermentations with lactobacilli in MRS broth supplemented with ferulic acid (FA) and coumaric acid, respectively. The degradation of the substrates and the accumulation of active aroma compounds were analyzed by using HPLC.

3.1.9.1 HPLC analysis of volatile aroma compounds after fermentations with lactobacilli in MRS supplemented with hydroxycinnamates

MRS broth supplemented with 0.5 mM ferulic acid and coumaric acid (stock solution of 250 –500 mM in ethanol), respectively, was fermented under the stated conditions in table 11 with lactobacilli for 72 hours. After 24, 48, and 72 hours of fermentation samples were drawn, filtered and analysed by using HPLC. The growth and purity of the fermentation flora was determined by pH-measurements and evaluation of the total plate count. The supernatants of the fermentations with broth supplemented with hydroxycinnamates and wort were analyzed by reverse phase HPLC. The analyses were performed with a Dionex Ultimate 3000 apparatus, equipped with a C18 RP column (Phenomenex Kinetex 100 mm x 4.6 mm, 2.6 µm), a column oven heated up to 30°C, and a UV-detector adjusted to following wavelengths: 260 nm, 280 nm, 300 nm, 320 nm. The flow rate was 1.3 ml/minute; the mobile phase consisted of eluent A (750 ml MeOH + 240 ml H_2O + 10 ml *o*-phosphoric acid) and eluent B (H_2O), the separation was achieved by following gradient program: 0-3 mins: 55% eluent B; 3-4.7 mins: 35% eluent B; 4.7-7.03 mins: 20% eluent B; 7.03 mins: 55% eluent B.

3.1.10 Wort fermentations with a combination of aroma active starter cultures

Subsequently, combinations of strains were defined for wort fermentations after evaluation of the PCR results in the screening approach for decarboxylases. Wort as a substrate is similar to liquid sourdough. The fermentations were performed with wort (approximately 16°P) from the “Bayerische Staatsbrauerei Weihenstephan” – Weihenstephaner Original beer. 30 ml wort was inoculated with 1 ml of an overnight grown pre culture, washed onetime with sterile tap water in a 100 ml Erlenmeyer flask. The Erlenmeyer flasks were wrapped in aluminum foil, closed with a cotton ball and incubated at 27°C without shaking for 120 hours (5 days). The accumulated aroma compounds were analyzed by HPLC at t=0, one day, two days, three days, and at the end of the fermentation period after five days. The growth and purity of the fermentation culture was confirmed by pH-measurements after 24 hours and at the end of the fermentation period; furthermore, the total plate count was evaluated after 24 hours of

fermentation. In parallel, a chemically acidified (pH 3.3-adjusted with lactate) control fermentation was also analyzed.

3.1.11 Sourdough fermentations with *Lactobacillus pontis* TMW1.1086

Sourdoughs were prepared with 20 g flour (type 550, brand Rosenmehl), 29 ml tap water, and 10 ml pre-washed culture of *L. pontis* supplemented with 0.1 g/l cycloheximide. Overnight grown cultures of *L. pontis* were centrifuged (5000 rpm, 10 mins, ambient temperature), and the supernatant was discarded. The cells were washed onetime with 10 ml tap water and finally resuspended in 10 ml tap water. The sourdough was inoculated with approx. 10^7-10^8 cfu/g dough. The sourdoughs were prepared in 200 ml Erlenmeyer flasks and incubated for 48 hours at 37°C. Dough samples were sent to FSI for active aroma compound analyses by using GC/MS.

3.2 RESULTS

3.2.1 Determination of predicted cinnamoyl esterase activity in lactobacilli

Screening of the selected lactobacilli in respect for predicted cinnamoyl esterase activity showed the formation of a clearing zone around the point of inoculation using agar plates supplemented with ethyl ferulate after three days of incubation. Remarkably, the thermophilic lactobacilli showed the highest activities, e.g. *L. acidophilus, L. johnsonii, L. helveticus, L. fermentum*, and *L. reuteri*, respectively. Two exceptions were found, *L. brevis* and *L. rossiae*, they showed also a certain activity after incubation at 30°C.

Table 17. Screening for cinnamoyl esterase activity in lactobacilli with mMRS agar plates supplemented with 0.1% ethyl ferulate and ethyl coumarate (final concentration). +++: high activity, > 20 mm diameter; ++: average activity, < 20 mm diameter; +: low activity, <10 mm diameter; -: no activity; n.d.: not determined. Red colored are the lactobacilli used for cloning and screening experiments.

Strain	**TMW**	**Cinnamoyl esterase activity**	
		Ethyl ferulate	**Ethyl coumarate**
B. subtilis	2.46	+++	n.d.
L. brevis	1.1326	++	+
L. nantensis	1.1265	+	n.d.
L. helveticus	1.1176	++	n.d.
L. casei	1.1250	-	n.d.
L. buchneri	1.1162	-	n.d.
L. alimentarius	1.62	+	n.d.
L. reuteri	1.106	++	n.d.
L. frumenti	1.103	+	n.d.
L. paralimentarius	1.256	-	n.d.
L. rossiae	1.162	++	n.d.
L. mindensis	1.1206	-	n.d.
L. pontis	1.1463	-	+
L. pontis	1.1086	++	n.d.
L. panis	1.1318	+	n.d.
L. farciminis	1.68	+	n.d.
L. gasseri	1.1173	++	n.d.
L. acidophilus	1.697	+++	n.d.
L. fermentum	1.890	++	n.d.
L. fructivorans	1.1253	-	n.d.
L. crispatus	1.143	-	n.d.
L. sanfranciscensis	1.53	+	n.d.
L. sanfranciscensis	1.1304	++	n.d.
L. spicheri	1.262	+	n.d.
L. plantarum	1.460	+	++
L. plantarum	1.468	++	++
L. johnsonii	1.1179	+++	n.d.

L. delbrueckii	1.1379	-	n.d.
L. perolens	1.501	-	n.d.
L. amylolyticus	1.1078	+	n.d.
L. amylovorus	1.653	+	n.d.
L. hilgardii	1.1298	-	n.d.
L. pentosus	1.10	+	n.d.
L. sakei	1.22	-	n.d.
L. zymae	1.943	++	n.d.
W. confusa	1.928	-	n.d.

The sizes of the clearing zone and therefore the esterase/hydrolase activities varied between 0 mm, e.g. *L. casei* TMW 1.1250, and 60 mm in screenings with *L. johnsonii* TMW1.1179. Some strains, e.g. *L. casei*, *L. buchneri*, *L. paralimentarius*, *L. mindensis*, *L. fructivorans*, *L. crispatus*, *L. delbrueckii*, and *L. perolens* exhibited no feruloyl esterase activity in the plate screening method with ethyl ferulate. Either their genetic background is not capable to hydrolyze ethyl ferulate or the enzyme is not transcribed. Furthermore, present phenolic compound concentrations could have an inhibitory effect on specific lactobacilli; their growth on the sensi-disc was prevented by the selected concentration of 0.1% ethyl ferulate.

3.2.2 Heterologously expression of predicted hydrolases/esterases from lactobacilli and characterizations of the recombinant enzymes

Based on the executed plate screenings from chapter 3.2.1 with different lactobacilli and ethyl ferulate as a substrate, the sequenced *Lactobacillus* genomes were examined for putative hydrolases/esterases able to release bonded hydroxycinnamates using database analysis (BlastX, EMBL-Heidelberg). Predicted cinnamoyl esterases of different lactobacilli were aligned for homologous regions by ClustalW. The conserved regions of predicted esterases from *L. helveticus* (Accession YP_001578032), *L. acidophilus* (YP194675), *L. gasseri* (YP815563), *L. plantarum* (YP_004890534), *L. reuteri* (YP_001842568), and *L. fermentum* (YP_001844134) could be clearly seen (see figure 25). The amino acid sequences of two cinnamoyl esterases from *L. johnsonii* were aligned together with the above mentioned sequences. Following sequences accordance could be seen on amino acid level for the *L. johnsonii* cinnamoyl esterase (Accession No. ADD11991, (31, 32)) by using the database BlastP:

- *L. gasseri* (YP 815563): 88% Identities; 95% Positives, 0% Gaps on 249 amino acids (aa).
- *L. acidophilus* (YP 194675): 70% Identities; 84% Positives, 0% Gaps on 244 aa.
- *L. helveticus* (YP_001578032): 70% Identities; 81% Positives, 2% Gaps on 248 aa.
- *L. plantarum* (YP_004890534): 52% Identities, 68% Positives, 2% Gaps on 238 aa.
- *L. reuteri* (YP_001842568): 49% Identities, 65% Positives, 2% Gaps on 249 aa.

- *L. fermentum* (YP_001844134): 50% Identities, 67% Positives, 2% Gaps on 222 aa.

```
L.johnsonii2        ------------METTIKRDGLNLHGLLEGTDKIENDTIAILMHGFKGDLGYDDSKILYA 48
L.reuteri           -----------MMEITIKRDGLKLYGLLEGTTTIKNDTIAILMHGFKGNLGYDDSKILYA 49
L.fermentum         ------------MEVAIKSAGLTLRGLLEGSNQVPNDRIAILMHGFKGDLGYTEENLLNQ 48
L.plantarum         ---------MTSMEFKIKRDGLALQARLE-TPAAPSSTLVILMHGFTADMGYDTTQFVPQ 50
L.johnsonii1        -----------MATITLERDGLQLVGTREEPFGEIYD-MAIIFHGFTANR---NTSLLKE 45
L.gasseri           MKLKKKKVGIYMATITIERDGLNLVGTREEPFGEIYD-MAIIFHGFTANR---NTPLLKE 56
L.acidophilus       -----------MSRITIERDGLTLVGDREEPFGEIYD-MAILMHGFTANR---NTPLLRQ 45
L.helveticus        -----------MSRITIERDGLTLVGDREEPFGEIYD-MAIIMHGFAANR---NTDLLRQ 45
                                    ::  ** * .  * .     . :.*::*** .:       ::

L.johnsonii2        LSHYLNDQGLPTIRFDFDGCGKSDGKFEDMTVYSEILDGIKILDYVRNTVKAKHIYLVGH 108
L.reuteri           LSHYLNQQGIPTLRFDFDGTGHSDGEFKDMTVFSEILDGMKIIDYAHTTMQAKKIYLIGH 109
L.fermentum         LAHRLNDQGLATLRFDFAGCGKSDGRFSDMTVLSELQDGMKIIDYARQEVQAKEIILVGH 108
L.plantarum         LAQALVAHGLAVLRFDFNGHGCSEGRFQDMTVINEIADAKAVLDEA-LTLHYDHIVLAGH 109
L.johnsonii1        IANSLRDENIASVRFDFNGHGDSDGKFENMTVLNEIEDANAILNYVKTDPHVRNIYLVGH 105
L.gasseri           IADELRDENIASVRFDFNGHGDSDGKFENMTVLNEIEDANAILNYVKTDPHVRNIYLVGH 116
L.acidophilus       IADNLRDENVASVRFDFNGHGESDGAFEDMTVCNEIADAQKILEYVRTDPHVRNIFLVGH 105
L.helveticus        IADDLRDENVASVRFDFNGHGESDGKFEDMTVCNEIADGKAILDYVRTDPHVRDIFLVGH 105
                    ::. *  ..:. :**** * * *:* *.:*** .*: *.  ::: .    :  .* * **
                                                          α/β subdomain
L.johnsonii2        SQGGVVASMLAGYYRDVIEKLALLSPAATLKSDALDGVCQGSTYDPTHIPETVNVS---- 164
L.reuteri           SQGGVVASMLAAYYRDIITKLVLLAPAATLKDDALKGVCQGSQYDPNHIPETVDVH---- 165
L.fermentum         SQGGVVASMLAAYYRDVIDKLVLLAPAATLKDDALIGTCQGTTYDPNHIPDYVTVG---- 164
L.plantarum         SQGGVVASMLAGYYPDVVDKLILMAPAATLKSDAQQGVLQGATYDPQHIPAYLNIRD--- 166
L.johnsonii1        SQGGVVASMLAGLYPDLIKKVVLLAPAATLKSDALEGNTQGVTYNPDHIPDRLPFKD--- 162
L.gasseri           SQGGVVASMLAGLYPDIIKKVVLLAPAATLKTDALNGSTQGVKYNPDHIPDRLPFKD--- 173
L.acidophilus       SQGGVVASMLAGLYPDIVKKVVLLAPAAQLKDDALNGDTQGATYNPEHIPAAIPFHG--- 162
L.helveticus        SQGGVVASMLAGLYPDVVKKVVLLAPAAQLKDDALRSNTQGATYDPNHIPDVVPLVGNKL 165
                    ***********. * *:: *: *::*** ** **  .  **  *:* ***  : .

L.johnsonii2        GFEVGGAYFRTAQLLPIYQTAEHYNRETLLIHGLADKVVSPNASRKFHTLLPKSELHLIP 224
L.reuteri           GFTVGGDYFRTAQLLPIYETAQHYSGPTLLIHGLADNVVSPEASKKYNVIMPNSELHLIP 225
L.fermentum         GFKVGGDYFRTAQLLPIYETAQHYAGPVLMIHGLADTVVDPKASQKYNVMYQNGVIHFLE 224
L.plantarum         GLKVGGFYLRTAQQLPIYEVAQQYAGSVTLIHVTADTVVSPQASEKYHEVYQHSQLHWVQ 226
L.johnsonii1        -LTLGGFYLRIAQQLPIYEVSAQFTKPVCLIHGTDDTVVSPNASKKYDQIYQNSTLHLIE 221
L.gasseri           -LTLGGFYLRIAQQLPIYEVSVHFTRPVCLIHGANDTVVSPDASKKYDQVYENSTLHLVE 232
L.acidophilus       -KKLGGFYLRTAQVLPIYEIAKHYTNPVSIIVGSNDQVVAPKYSKKYDEVYENSELHMVP 221
L.helveticus        GMKLGGFYLRTAQVLPIYEVSQCFTRPVSVIAGTNDQVVDPKYAKKYDEVYENSELHMIP 225
                       :** *:* ** ****: :  :   . :*    * ** *. :.*:. :  :. :* :

L.johnsonii2        DEGHMFNGK--NRPEVLKLVGEFLIK---- 248
L.reuteri           EEGHMFNGS--RRQEILELVANFLKN---- 249
L.fermentum         GASHQLRGDGDQRETTLQLVADFLN----- 249
L.plantarum         DGGHRFSGD--ARATAIQLALAAFK----- 249
L.johnsonii1        GADHCFSDS--YQKNAVNLTTDFLQNNNAF 249
L.gasseri           GADHSFTDT--YQKTAADLTAEFLQDNNTF 260
L.acidophilus       DADHSFTGQ--YKDSAVDLTAEFLKP--LF 247
L.helveticus        NADHRFSGG--YKDMAADLTAQFLKP--AF 251
                      .* : .    :    .*.   :
```

Figure 25. Alignment of predicted cinnamyol esterases from different genome sequenced lactobacilli – all six proteins, besides the *L. johnsonii* ones were heterologously expressed in *E. coli* and characterized. An asterix indicates a position with a fully conserved amino acid residue; a colon indicates a position with a fully conserved strong group and a period indicates a position with a fully conserved weaker group. Red colored is the consensus sequence motif for carboxyl esterases Gly-x-Ser-x-Gly (x represents an arbitrary amino acid residue); blue marked is the presumed catalytic triad (Ser – His – Asp). Grey shaded: α/β-subdomain; brown marked are the amino acids of the hydrophobic cavity; green marked are amino acids responsible for substrate binding in the α/β-subdomain of the cinnamoyl esterases.

The sequence motif GHSQG is also present in a cinnamoyl ester hydrolase from the ruminal bacterium *Butyrivibrio fibrisolvens* El4 (15, 16). DNA sequences for predicted esterases in the genomes from *L. brevis* ATCC367 and *L. johnsonii* (see Accession No. ADD11991 and ADD11992 correspondent to (31, 32)) showed also a similar motif as the mentioned above. However, the specific genes were cloned in *E. coli* TOP10, but the recombinant proteins exhibited no activity after separation and purification; therefore, the genetic background for the clearing zones in plate screening methods with ethyl ferulate and these strains (see table 17) could not be biochemically verified.

All aligned amino acid sequences showed distinct features of cinnamoyl esterases; the inserted α/β-subdomain was previously identified as a prominent structure necessary for phenolic ring building and the formation of the catalytic pocket. The active site is formed by the classical triad of Ser, His, and Asp (21), the role of His is to deprotonate Ser so that Ser can perform a nucleophilic attack on the carbon atom of the carbonyl group of the hydroxycinnamates, while Asp stabilizes the protonated His (32). The 4-hydroxyl group (ethyl ferulate, ferulic acid, and caffeic acid) and 3-hydroxyl group (caffeic acid) of the aromatic ring of the substrates are hydrogen bonded to Asp and Tyr, respectively (green marked, see figure 25). Moreover, Asp and Gln from the inserted α/β-subdomain are important in recognizing the caffeic and feruloyl esters (32). The structure of the α/β-subdomain is also important for holding the phenolic ring of the phenolic esters in the correct position, site-directed mutagenesis of the subdomain showed the assumption; no activity was detected with any of the phenolic esters as a substrate for the predicted cinnamoyl esterase of *L. johnsonii* (32). SignalP program results showed that there were no predicted signal peptides available for all above listed amino acid sequences (data not shown).

3.2.2.1 Verification of the heterologous expression using SDS page gel and activity determination using mMRS plates supplemented with ethyl ferulate

As an example, the cloning success, and therefore, the heterologous expression of the predicted cinnamoyl esterase of *L. plantarum* TMW1.460 was shown after purification using FPLC and separation of the protein rich fraction. SDS gel was prepared with a molecular marker (100 bp ladders), crude cellular extract of *L. plantarum*, and the predicted cinnamoyl esterase of *L. plantarum* (data not shown). The definitive mass of the enzymes was determined with the help of ExPASy (Swiss Institute of Bioinformatics). The heterologously

expressed predicted cinnamoyl esterases (see chapter 3.2.2) from the lactobacilli had similar p*I* ranging from 5.04 – 5.78, and theoretical Mw ranging from 27.1-28.9 kDa.

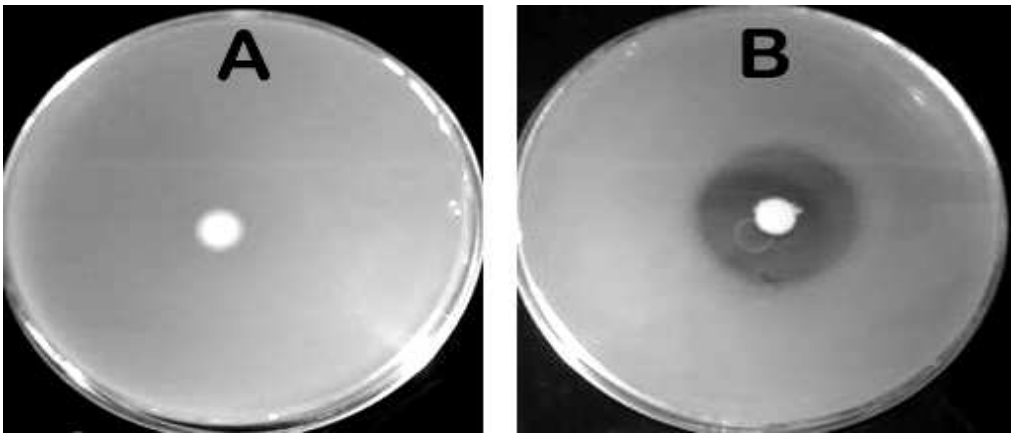

Figure 26. Plate assay with LB agar and supplemented ethyl ferulate (0.1%). (A) Control with *E. coli* TOP10 hosting the vector pBAD/*Myc*-His B without insert; (B) *E. coli* TOP10 with pBAD/*Myc*-His B and the cloned predicted cinnamoyl esterase from *L. plantarum* TMW1.460.

The plate assay was performed with LB agar plates supplemented with ethyl ferulate (final concentration 0.1%), 75 ppm ampicillin and arabinose (final concentration 1 mM). The sensi-discs were wetted with 20 µl *E. coli* TOP10 cells and incubated aerobically at 37°C for two days. The cloning success of the predicted cinnamoyl esterase from *L. plantarum* TMW1.460 (exemplary for all cloning approaches) could be seen in figure 26, due to an intact recombinant cinnamoyl esterase a clearing zone around the point of inoculation was observed.

3.2.2.2 Enzyme kinetics of the heterologously expressed cinnamoyl esterases from lactobacilli and pH-dependent activity

Evaluations of the relative enzymatic activity of the recombinant proteins were performed with the substrate para-nitrophenyl acetate (p-NP-C2); the substrate was also used in other studies related to activity determination of predicted cinnamoyl esterases (31, 45). The enzymatic reactions were performed in 100 mM sodium phosphate buffer with a pH-value of 7.0 at ambient temperature. The recombinant esterases from lactobacilli exhibited diverse affinities to p-NP-C2. The highest affinity towards the substrate was achieved by the esterase of *L. plantarum* TMW1.460, the lowest affinity was measured for the esterase of *L. reuteri* TMW1.106 (see table 18). The highest reaction velocities were achieved of the heterologous expressed cinnamoyl esterases from *L. gasseri* TMW1.1173 and *L. helveticus* TMW1.1176 in comparison to the other lactobacilli predicted by the Michaelis-Menten equation. The K_m-values, and therefore the affinity of the heterologous expressed esterases towards the substrate

p-NP-C2, and the maximum reaction velocities V_{max} of the esterases were not correlated to each other.

Table 18. Enzyme kinetics of the recombinant cinnamoyl esterases in reactions with the substrate p-nitrophenyl acetate

Esterase of strain	**K_m [µmol*l^{-1}]**	**V_{max} [µmol*mg^{-1}*min^{-1}]**
L. plantarum TMW1.460	5.25	91.21
L. gasseri TMW1.1173	7.63	134.35
L. fermentum TMW1.890	8.54	76.98
L. acidophilus TMW1.697	17.25	46.30
L. helveticus TMW1.1176	21.67	129.67
L. reuteri TMW1.106	28.21	4.57

3.2.2.3 Characterization of the pH-dependent activity of the cinnamoyl esterases

The pH optimum related to enzymatic activity of the heterologous expressed cinnamoyl esterases was determined in a pH range of 4.0–9.0. Different buffer systems were used to cover the range (see chapter 3.1.1.3); p-NP-C2 was also used as a substrate in the pH-dependent characterization of the recombinant proteins. The relative activities of all cinnamoyl esterases were increased from a pH-value of 4.0 to 7.0; the highest activity at a pH-value of 7.0 was achieved by the esterase of *L. fermentum* TMW1.890.

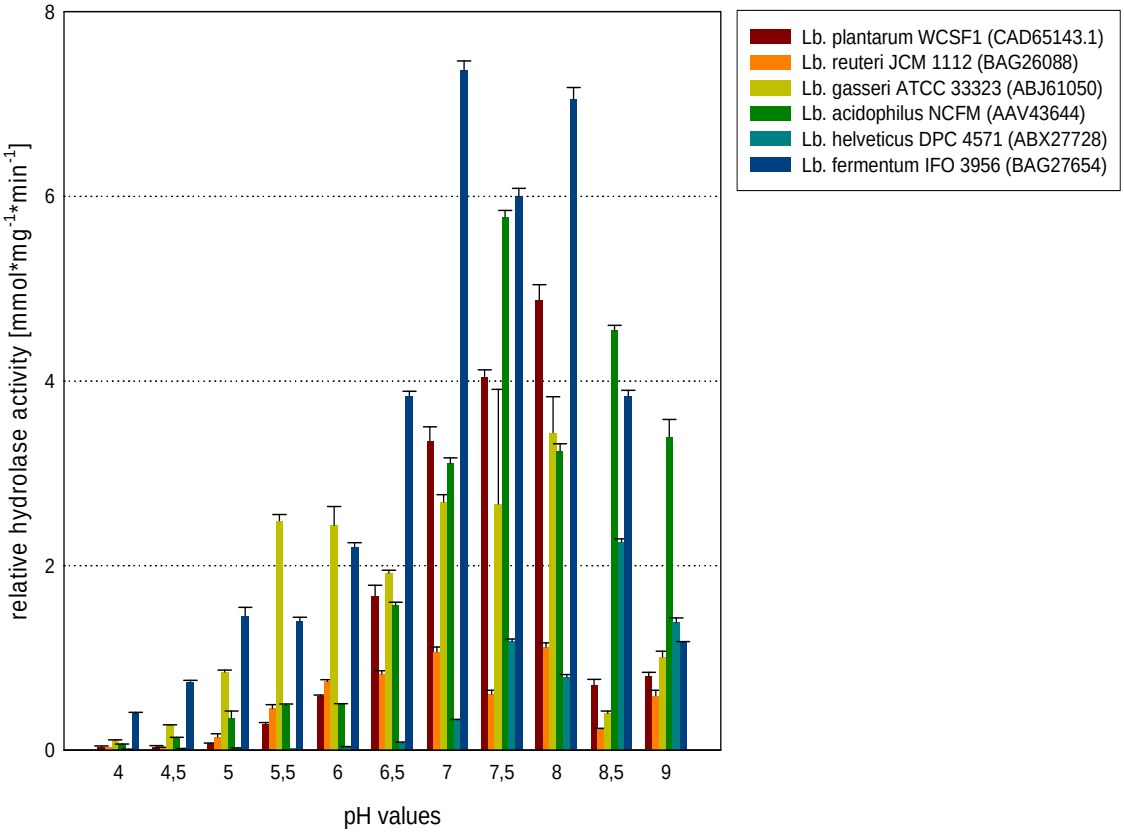

Figure 27. Determination of the pH optimum of the recombinant cinnamoyl esterases.

Generally, the relative activities of all recombinant cinnamoyl esterases showed an optimum between a pH-value of 7.0–8.0; with the exception of the esterase from *L. helveticus* TMW1.1176, their pH optimum was located at a pH-value of 8.5. At the higher pH-value of 9.0 all cinnamoyl esterases showed a decreased relative activity, nonetheless the activity of the esterase from *L. acidophilus* TMW1.697 remained on a remarkable high level. The relative activity of the esterase from *L. gasseri* TMW1.1173 was already at a constant high level beginning with pH 5.5. The activities of the esterases from *L. reuteri* TMW1.106 and *L. helveticus* TMW1.1176, respectively, showed overall the lowest activities throughout the pH range from 4.0–9.0. This fact could be explained with the lowest affinity of the esterases from *L. reuteri* and *L. helveticus* towards the substrate p-NP-C2 (see table 18).

3.2.2.4 Characterization of the substrate side-chain length-dependent activity of the cinnamoyl esterases

Three different substrates with diverse side chain lengths were used to determine the relative enzymatic activity of the cinnamoyl esterases; all reactions were performed in sodium phosphate buffer with a pH-value of 7.0 at ambient temperature. Following substrates were used: p-nitrophenyl acetate (p-NP-C2), p-nitrophenyl butyrate (p-NP-C4), and p-nitrophenyl caprylate (p-NP-C8).

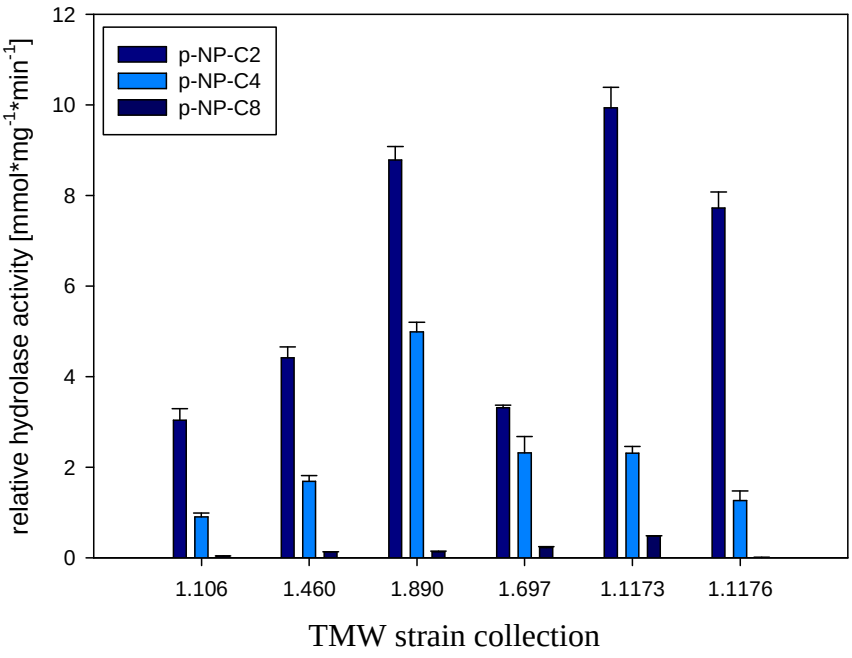

Figure 28. Determination of the substrate side chain length-dependent activity of the heterologously expressed cinnamoyl esterases. 1.106 = *L. reuteri*, 1.460 = *L. plantarum*, 1.890 = *L. fermentum*, 1.697 = *L. acidophilus*, 1.1173 = *L. gasseri*, 1.1176 = *L. helveticus*.

All heterologously expressed cinnamoyl esterases showed a substrate side chain length-dependent activity. When substrates were used with enlarged side chain lengths, e.g. p-NP-C8 the activities were drastically decreased. The highest activity towards p-NP-C8 was measured for the esterase from *L. gasseri* TMW1.1173; nonetheless the activity was a fraction in comparison with the relative activity towards p-NP-C2. Interestingly, the relative activity towards p-NP-C2 and p-NP-C4 was comparable in reactions with the esterase from *L. acidophilus* TMW1.697.

3.2.2.5 Characterization of the temperature-dependent activity of the cinnamoyl esterases

For the determination of a temperature-dependent activity only the esterases from *L. acidophilus* TMW1.697, *L. plantarum* TMW1.460, *L. gasseri* TMW1.1173, and *L. reuteri* TMW1.106 were selected; the activities were evaluated in a temperature range from 20–60°C (data not shown). The cinnamoyl esterase from *L. gasseri* showed a maximum activity at 45°C; then a drastically decrease in activity was evaluated beginning at 50°C to 60°C. The esterase from *L. plantarum* showed almost same activities in the temperature range from 20–35°C, following a constant decrease at higher temperatures. The temperature-dependent activities of the esterases of *L. acidophilus* and *L. reuteri* showed virtually the same course; a constant decrease in acitivity was observed from 20–60°C.

3.2.3 Fermentations with different lactobacilli in broth supplemented with cinnamic acid derivatives

The metabolisms of phenolic compounds during three day fermentations with lactobacilli were visualized using thin layer chromatography. For the evaluation following strains of the genus *Lactobacillus* were used: *L. panis* TMW1.1318, *L. acidophilus* TMW1.697, *L. sanfranciscensis* TMW1.53, *L. fermentum* TMW1.890, *L. pontis* TMW1.1086, *L. plantarum* TMW1.460, *L. reuteri* TMW1.106, *L. gasseri* TMW1.1173, and *L. johnsonii* TMW1.1179. The fermentations in mMRS were started after supplementation of the particular substrate (final concentration of 2 mM); the analyses were conducted with methyl caffeate, methyl coumarate, and ethyl ferulate. Subsequently, the samples were processed as stated in chapter 3.1.4. For example, only the results are shown for the fermentations with methyl caffeate as the substrate, because the results are representative for ethyl ferulate and methyl coumarate as well.

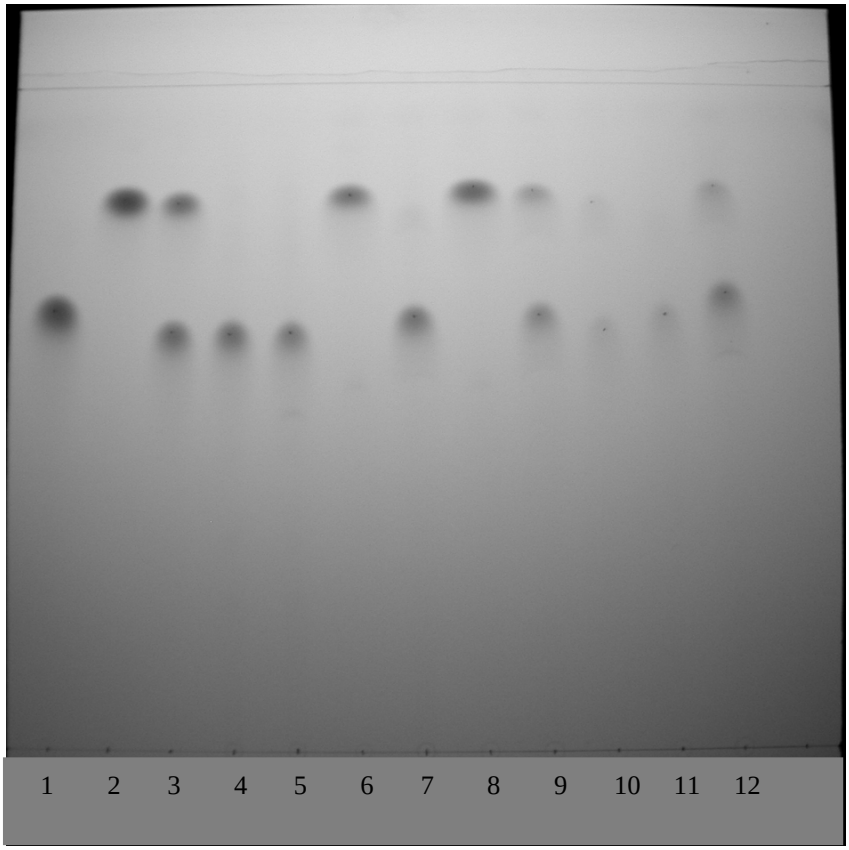

Figure 29. Thin layer chromatography analysis (TLC) of three day fermentations with lactobacilli in mMRS supplemented with methyl caffeate, visualized at 254 nm UV-light. 1: Standard, caffeic acid; 2: Standard, methyl caffeate; 3: Standard-Mix, caffeic acid and methyl caffeate; 4: *L. panis* TMW1.1318; 5: *L. acidophilus* TMW1.697; 6: *L. sanfranciscensis* TMW1.53; 7: *L. fermentum* TMW1.890; 8: *L. pontis* TMW1.1086; 9: *L. plantarum* TMW1.460; 10: *L. reuteri* TMW1.106; 11: *L. gasseri* TMW1.1173; 12: *L. johnsonii* TMW1.1179.

The degradation of the substrate methyl caffeate after three days of fermentation with different lactobacilli is shown in figure 29. *L. panis* TMW1.1318, *L. acidophilus* TMW1.697, and *L. fermentum* TMW1.890 exhibited the highest activity towards the substrate; almost the whole substrate was degraded to caffeic acid. A lower affinity towards the substrate methyl caffeate was shown in fermentations with *L. plantarum* TMW1.460, *L. reuteri* TMW1.106, *L. gasseri* TMW1.1173, and *L. johnsonii* TMW1.1179, respectively. No liberation of caffeic acid could be seen in fermentations with *L. sanfranciscensis* TMW1.53 and *L. pontis* TMW1.1086. As mentioned before, these results are representative also for the other fermentations with the substrates ethyl ferulate and methyl coumarate (data not shown). The decreased activity, e.g. *L. plantarum* and showing of none activity, e.g. *L. sanfranciscensis* could be either derived from an inhibitory effect of the phenolic compound against the fermentation flora, or the fermentation organism is genetically not equipped with the specific gene of interest. Moreover, the predicted enzyme could not be expressed under the perspective fermentation conditions of the above indicated approach.

3.2.4 Screening of lactobacilli for α-L-arabinofuranosidase genes using PCR

In this chapter a screening was performed for key enzymes in lactobacilli, e.g. α-L-arabinofuranosidase involve in the release of bonded ferulic acid out of arabinoxylan (see chapter 1 and figure 23, respectively). The evaluation of the existence of α-L-arabino-furanosidase genes in *Lactobacillus* strains was determined by PCR using degenerated primers AFN-I/AFC and AFN-II/AFC, respectively (44). The screening by PCR with genomic DNA and the stated primers was conducted with following strains: *Bifidobacterium lactis* TMW2.462, *L. sanfranciscensis* TMW1.1304, *L. brevis* TMW1.1326 (ATCC 367), *L. sakei* TMW1.22, *L. pontis* TMW1.1086, *L. plantarum* TMW1.460, *L. sanfranciscensis* TMW1.53, and *L. pentosus* TMW1.10. Arabinofuranosidases are playing a major physiological role in Bifidobacteria (35); therefore, *Bifidobacterium lactis* TMW2.462 was selected as a reference in the PCR.

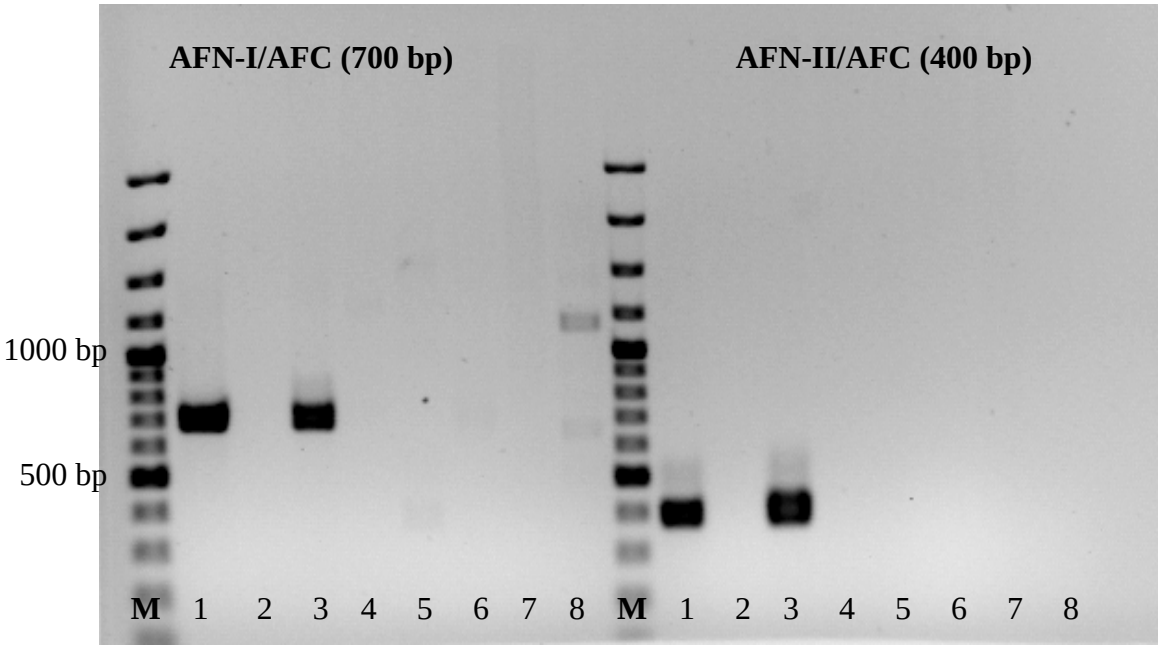

Figure 30. Screening for arabinofuranosidase genes in lactobacilli using PCR. Predicted amplificate sizes of AFN-I/AFC: 700 bp, AFN-II/AFC: 400 bp. M= 100 bp standard. Lane 1: *B. lactis*, Lane 2: *L. sanfranciscensis* TMW1.1304, Lane 3: *L. brevis*, Lane 4: *L. sakei*, Lane 5: *L. pontis*, Lane 6: *L. plantarum*, Lane 7: *L. sanfranciscensis* TMW1.53, Lane 8: *L. pentosus*.

The screening confirmed the unique occurrence of α-L-arabinofuranosidase genes in *Lactobacillus brevis* TMW1.1326; the amplificates were also seen in the reference *Bifidobacterium lactis*. Besides, all other evaluated strain DNA's showed no PCR product in the desired size.

```
Enterococcus      ---MEATMLISSVKRVGKVDPRLYSSFIEHMGRAVYEGIYQPDHPSANKSGFRQDVIHLV 57
Geobacillus       MATKKATMIIEKDFKIAEIDKRIYGSFIEHLGRAVYGGIYEPGHPQADENGFRQDVIELV 60
Ara-1-brevis      ---MQGKLTVDPASQIAKIDDRVYSALIEHLGRSVYDGLYQPNHPQSDQDGFRQDVIDAV 57
Ara-2-brevis      ---MQGSTKIQIQDVVAPIDRRLYGAFIEQLGRAVYTGIYQPDHPTADSDGFRTDVIDAI 57
                      :..  :.    :. :* *:*.::**::**:** *:*:*.** ::..*** ***. :
                           AFN-I
                           ──────►

Enterococcus      KELNIPMIRYPGGNFVSGFRWEDSIGPIANRPRRLDLAWRTIETNEVGIHEFYNWCQHVG 117
Geobacillus       KELQVPIIRYPGGNFVSGYNWEDGVGPKEQRPRRLDLAWKSVETNEIGLNEFMDWAKMVG 120
Ara-1-brevis      KSLNIDLIRYPGGNFVSGFNWEDSIGPKSTRPTRLDLAWRSIETNQFGLHEFMKWTQKTG 117
Ara-2-brevis      KTLNVPLIRYPGGNFLSQYRWEDGIGPKSQRPVRLDLAWRELETNQFGLHEFMRWADKVN 117
                  * *:: :********:* :.***.:**   ** ******: :***:.*::**  * . ..
                                                                AFN-II
                                                                ──────►

Enterococcus      AEINMAVNLGTRGVDAARNLVEYCNFPKGTYWSDLRRSNGQESPFGIKTWCLGNEMDGPW 177
Geobacillus       AEVNMAVNLGTRGIDAARNLVEYCNHPSGSYYSDLRIAHGYKEPHKIKTWCLGNEMDGPW 180
Ara-1-brevis      TRPDMAVNLGSRGIDAARNLVEYCNFSGGTYWSDLRKQNGAEEPFNIKTWCLGNEMDGDW 177
Ara-2-brevis      AVPNMAVNLGTRGIQAAADLIEYCNFPKGTYLSDLRRQNGAEHPFAIKTWCLGNEMDGPW 177
                  :  :******:**::** :*:****.. *:* ****  :* : *. ************ *


Enterococcus      QIGHKTAEEYGRLAEETAKAMKLVDDSIEVVLCGSSNRQMPTFGDWELTVLDQAYDQIDY 237
Geobacillus       QIGHKTAVEYGRIACEAAKVMKWVDPTIELVVCGSSNRNMPTFAEWEATVLDHTYDHVDY 240
Ara-1-brevis      QIGHKTADEYGRLAHETAKVMRLVDPSIDLVVSGSSTREMATFGSWEETVLDHTYDDVDY 237
Ara-2-brevis      EIGAKSAGEYAHLANETAKAMRRVDDTLELVACGSSSMDNPTFGNWEETVLDACYDNVDY 237
                  :** *:* **.::* *:**.*: ** ::::* .***. : .**..** ****  **.:**
                                                                AFC
                                                                ◄──────

Enterococcus      LSLHQYYGNQKND----LSHYLARSLDMDRFISEVTAMCDTVKAKKHSKKQINLSFDEWN 293
Geobacillus       ISLHQYYGNRDND----TANYLALSLEMDDFIRSVVAIADYVKAKKRSKKTIHLSFDEWN 296
Ara-1-brevis      LSLHRYYDNEEND----LANFLAKSIDFDEFISGIVAVCDAVKARKHSHKTLNLALDEWN 293
Ara-2-brevis      LSLHRYYGYYNDDDPTELDNFLGKNHDLDDFIKGVVAMCDAVKARKRSTKTINLSFDEWN 297
                  :***:**.  .:*      ::*. . ::* **  :.*:.* ***:*:* * ::*::****

Enterococcus      IWYHSAEQDEQAAPWQIAPPLLEDNYNFEDALLLGCLLITLLKHADRVKIACLAQLVNVI 353
Geobacillus       VWYHSNEADKLIEPWTVAPPLLEDIYNFEDALLVGCMLITLMKHADRVKIACLAQLVNVI 356
Ara-1-brevis      VWYHSHKQDDETAPWQQAPHLLEDHYNFEDALMVGTMLITLLKHADRVKIACLAQLVNVI 353
Ara-2-brevis      VWYHSNDADTQVTPWQVGPHLLEDIYNFEDALLIGSLLMTLLRNADRVKIACLAQLVNVI 357
                  :**** . *    **  .* **** *******:* :*:**:::**************

Enterococcus      APIRTETNGEAWRQTIYYPFMQAAVYGQGEVLSPQIRSANYATEDFEEVPYLESIAVIND 413
Geobacillus       APIMTEKNGPAWKQTIYYPFMHASVYGRGVALHPVISSPKYDSKDFTDVPYLESIAVYNE 416
Ara-1-brevis      APIMTDEHG-IWLQSIFFPFLQISKYGRGIALTPQQTATTYDSKDFKDVPYLDSLAVYNS 412
Ara-2-brevis      APIMTDENGGVWRQSIFYPFMQVANYGQGVVLTDHSTSPTYNSREFTDVAYLDTVTTYDA 417
                  *** *: :*  * *:*::**:: : **:* .*     :..* :.:* :*.**::::. :

Enterococcus      K--ELVIFAVNR-GEEEMNLTVHLSELSLEGVIDFSEMSGFDTKATNLPKSEQVKPSHST 470
Geobacillus       EKEEVTIFAVNRDMEDALLLECDVRSFEDYRVIEHIVLEHDNVKQTNSAQSSPVVPHRNG 476
Ara-1-brevis      ENHEIVIFAENK-GNTTMDFQTDLHDLNAQTVIEATQFCGYDIKQTN--ENQQMALHPNS 469
Ara-2-brevis      ATQTVTVFAENKHQTETLDFELNFGELMIDRLLDATQFYGYDVKADNRDGHMQLQPLP-- 475
                      :.:** *:     : :  .. .:    :::   :   : *  *      :

Enterococcus      NVKAEDGKINCLLKPLSWNVVRCRLSDEGGTKNER 505
Geobacillus       DAQLSDRKVSATLPKLSWNVIRLGKR--------- 502
Ara-1-brevis      NVHLSDNTLTASLQPLSWNMFRLHVTH-------- 496
Ara-2-brevis      -ARTDTHHAHTKLQPLSWNVLRFKLRD-------- 501
                   .: .       *  ****:.*
```

Figure 31. Alignment of α-L-arabinofuranosidase amino acid sequences from different micro-organisms, *Enterococcus faecium* (EJY49498), *Geobacillus stearothermophilis* (ACE73682), and *L. brevis* ATCC 367 (YP_795848 and YP_796302). Grey shaded are the regions from the derived primers AFNI, AFNII, and AFC used in this PCR approach. An asterix indicates a position with a fully conserved amino acid residue; a colon indicates a position with a fully conserved strong group and a period indicates a position with a fully conserved weaker group.

Harboring α-L-arabinofuranosidase genes inside the genome is not widespread among lactobacilli. Recently, predicted enzymes were also seen in the genomes of *Lactobacillus oris* PB013-T2-3, *Lactobacillus parafarraginis* F0439, *Lactobacillus kisonensis* F0435, and *Lactobacillus buchneri* NRRL B-30929.

3.2.5 Heterologously expression of a predicted α-L-arabinofuranosidase and xylosidase from *L. brevis* TMW1.1326 and characterization of the recombinant enzymes

The interaction of both enzymes is the basis for the liberation of ferulic acid out of the arabinoxylan backbone. The genome of *L. brevis* TMW1.1326 (ATCC 367) is harboring two genes for α-L-arabinofuranosidase, and two β-xylosidase genes. In each case one specific gene for α-L-arabinofuranosidase and β-xylosidase was cloned in *E. coli* and the recombinant enzymes were further characterized. Following sequence accordance could be seen on amino acid level for the *L. brevis* α-L-arabinofuranosidase (YP_796302) by using the database BlastP:

- *L. oris* (ZP_07729105): 69% Identities; 80% Positives, 1% Gaps on 502 amino acids (aa).
- *Carnobacterium* (YP_004374029): 59% Identities; 75% Positives, 4% Gaps on 509 aa.
- *L. brevis* (YP_795848): 59% Identities; 75% Positives, 3% Gaps on 503 aa.
- *L. parafarraginis* (ZP_09393561): 61% Identities, 75% Positives, 2% Gaps on 500 aa.

According to the current classification of glycosyl hydrolases the enzymes could be classified as α-L-arabinofuranosidases. The enzyme catalyzes the hydrolysis of non-reducing terminal α-L-arabinofuranosidic linkages in L-arabinose-containing polysaccharides [EC: 3.2.1.55] (NCBI, Conserved domains). Following sequence accordance could be seen on amino acid level for the *L. brevis* β-xylosidase (YP_794569) by using the database BlastP:

- *W. confusa* (ZP_10258500): 71% Identities; 83% Positives, 0% Gaps on 552 aa.
- *L. oris* (EGS35772): 68% Identities; 81% Positives, 1% Gaps on 552 aa.
- *L. antri* (ZP_05746624): 68% Identities; 80% Positives, 1% Gaps on 552 aa.

According to the current classification of glycosyl hydrolases the enzymes could be classified as β-D-xylosidase in the glycosyl hydrolase family 43. The glycosyl hydrolase family 43 includes mostly enzymes that have been characterized to have β-1,4-xylosidase activity [EC: 3.2.1.37]. They are part of an array of hemicellulases that are involved in the final breakdown of plant cell-wall whereby they degrade xylan (NCBI, Conserved domains). The cloning of the α-L-arabinofuranosidase gene was performed after obtaining a PCR product with primers arabino-Klon-for/arabino-Klon-rev (see table 12) harboring *Xho* I and *Xba* I restriction sites and isolated genomic DNA as a template; digestion and ligation into the *Xho* I and *Xba* I restriction sites of vector pBAD/Myc-His B resulted in a replicating integration vector, which

was cloned in electro competent cells of *E. coli* TOP10 by electroporation (see also chapter 5.1.8). The constructed vector harboring the cloned gene was isolated with the PegGold plasmid miniprep Kit I for DNA sequencing. The expression and purification of the heterologously expressed enzyme was performed as stated in chapter 3.1.5. The identical cloning process was conducted for the heterologous expression of the xylosidase from *L. brevis*; with primers b-xyl-1-Klon-for/b-xyl-1-Klon-rev (see table 12) harbouring *Nco* I and *Xba* I restriction sites and isolated genomic DNA a PCR product was obtained. This time, the digestion and ligation was conducted with the help of the *Nco* I and *Xba* I endonucleases. The cloning success and, therefore, the heterologous expression of the predicted enzymes of *L. brevis* TMW1.1326 is shown after purification using FPLC and separation of the protein rich fraction. SDS gel was prepared showing a molecular marker, crude cellular extracts of *L. brevis*, and the predicted purified enzymes.

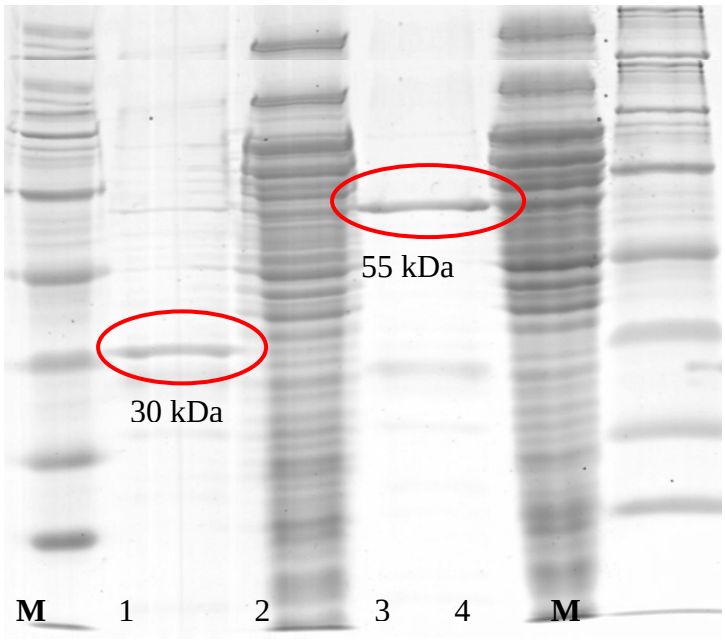

Figure 32. SDS-PAGE gel of the heterologously expressed enzymes. M= molecular weight marker. Lane 1: Purified xylosidase, Lane 2: crude cellular extract of *E. coli* TOP10 (xylosidase), Lane 3: Purified α-L-arabinofuranosidase, Lane 4: crude cellular extract of *E. coli* TOP10 (arabino-furanosidase).

3.2.5.1 Characterization of the temperature and pH-dependent activity of the α-L-arabinofuranosidase and xylosidase from L. brevis TMW1.1326

The effects of temperature and pH-value on the enzymes activities were determined with p-NP-ara and p-NP-D-xyl as the substrates according to chapter 3.1.7. The activities at the optimal temperature and optimal pH-value were defined as 100%. The recombinant arabinofuranosidase showed maximum of activity between a pH-value of 6-7, and at 45°C.

The xylosidase of *L. brevis* showed an activity maximum in the pH-range of 6.5-7.5, and at 25°C. Remarkably, the heterologous expressed arabinofuranosidase showed optimal activity at 45°C, whereas the xylosidase exhibited almost none activity towards the substrate already at a temperature of 40°C. The xyl-1 enzyme hydrolyzed p-nitrophenol-β-D-xylopyranoside with an apparent K_m of 9.05 µmol x L^{-1} and V_{max}-value of 11.82 µmol x mg· min^{-1}.

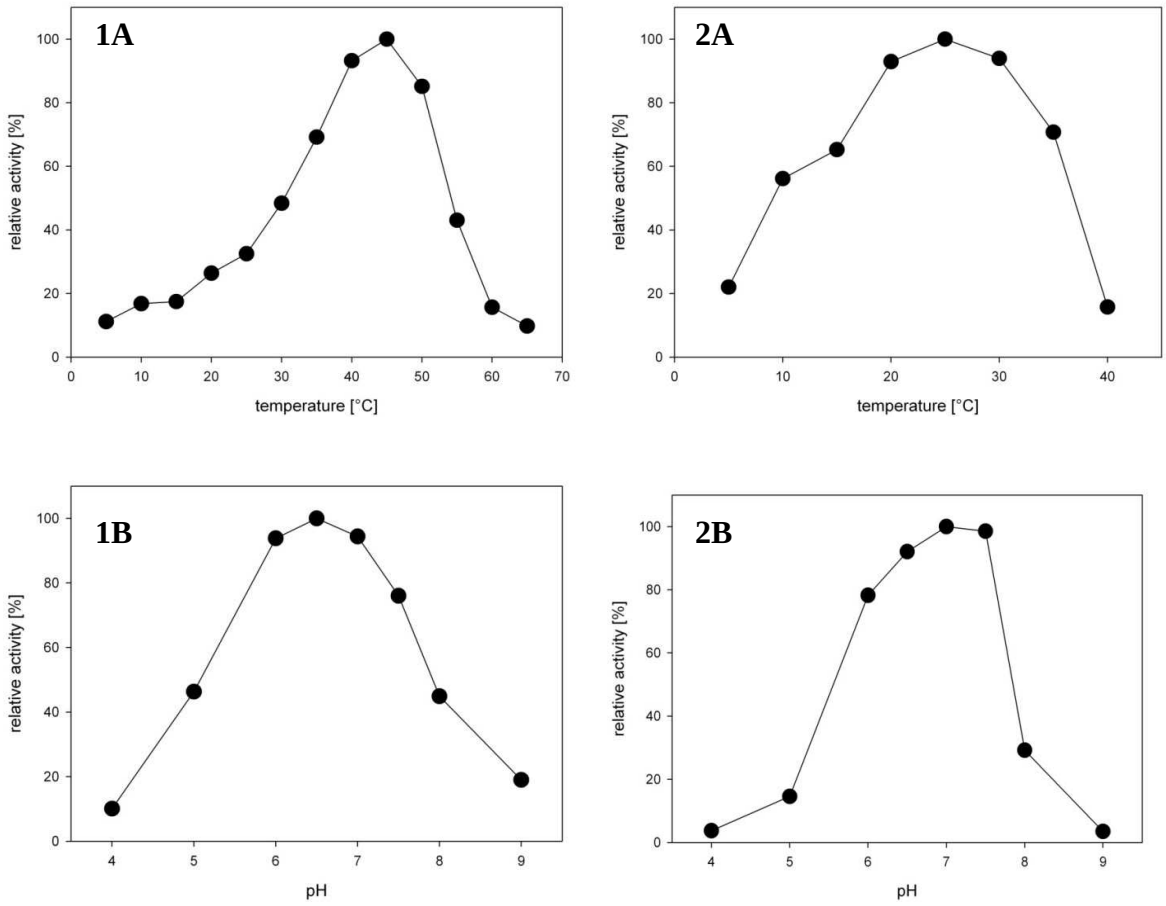

Figure 33. Temperature -and pH-dependent relative activities of the recombinant enzymes. 1A: Temperature-dependent activity of arabinofuranosidase, 1B: pH-dependent activity of the arabino-furanosidase, 2A: Temperature-dependent activity of the xylosidase, 2B: pH-dependent activity of the xylosidase

3.2.5.2 TLC analysis of the heterologously expressed enzymes against branched poly-saccharides

Substrate specificity was analyzed with TLC, showing that both enzymes were active in a coupled reaction against water-soluble wheat arabinoxylan. Thin layer chromatography analysis (TLC), visualized the activities of the recombinant enzymes according to chapter 3.1.7.1. Arabinose and xylose were released in reactions with the enzymes and arabinan and xylan as a substrate, respectively. Both enzymes exhibited also activity when wheat

arabinoxylan was used; therefore, it could be assumed that both enzymes are able to release pentoses out of branched polysaccharides.

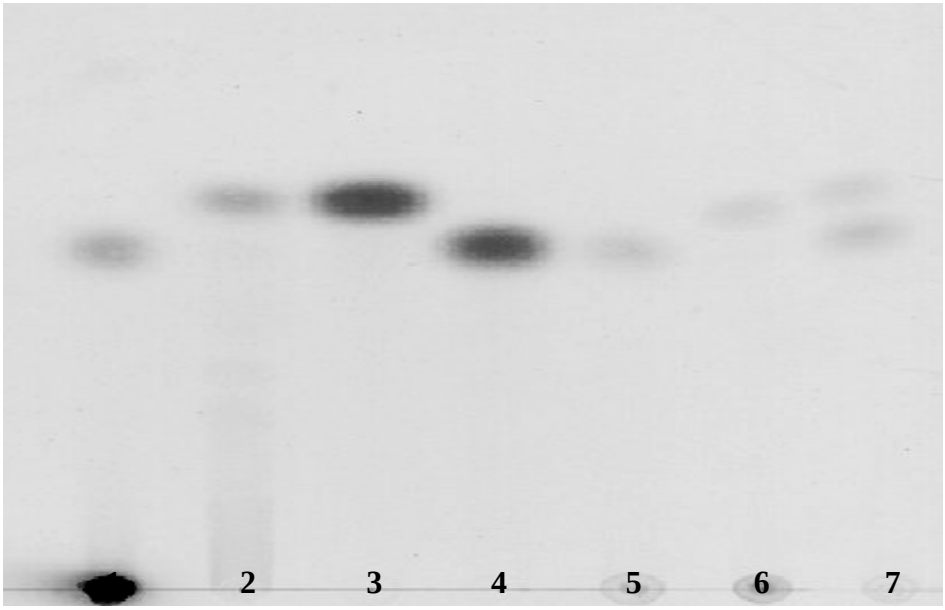

Figure 34. TLC analysis of the activity of the recombinant enzymes against branched polysaccharides. Lane 1= Arabinan + ara-2; Lane 2= Xylan + xyl-1; Lane 3= Xylose (10mM); Lane 4= Arabinose (10mM); Lane 5= Wheat arabinoxylan + ara-2; Lane 6= Wheat arabinoxylan + xyl-1; Lane 7= Wheat arabinoxylan +ara-2 +xyl-1. The relative R_f-values were measured for each sample.

3.2.6 Screening of phenolic acid decarboxylase genes using PCR and verification of decarboxylase activity in lactobacilli

The introduction of this chapter concerned with the enzymatic conversion of ferulic acid to 4-vinylguaiacol with the help of phenolic acid decarboxylase (see figure 23). In additional studies performed at our department, a decarboxylase-PCR product with the selected primers according to de las Rivas (17) was obtained for the *Lactobacillus plantarum* strains (TMW1.460, and TMW1.468), *Lactobacillus pontis* (TMW1.1068, TMW1.1463, and TMW1.1469), and *Lactobacillus brevis* TMW1.1326. None of the *Lactobacillus sanfrancis-censis* strains (TMW1.52, TMW1.53, and TMW1.1304) showed a PCR amplificate. In this approach, only four different lactobacilli were tested in PCR screenings for decarboxylase genes with genomic DNA and degenerated primers. *L. plantarum* TMW1.460 showed an amplificate for a PDA and PDC gene, *L. sakei* TMW1.22 only for a PDC gene, *L. fermentum* TMW1.890 and *L. sanfranciscensis* TMW1.53 exhibited no specific PCR product. Both PCR screenings indicated that the examined *Lactobacillus sanfranciscensis* strains harboring none phenolic acid decarboxylase gene. Moreover, two different sequences for phenolic acid decarboxylases were obtained in the screening studies with *L. pontis* TMW1.1086 (data not shown).

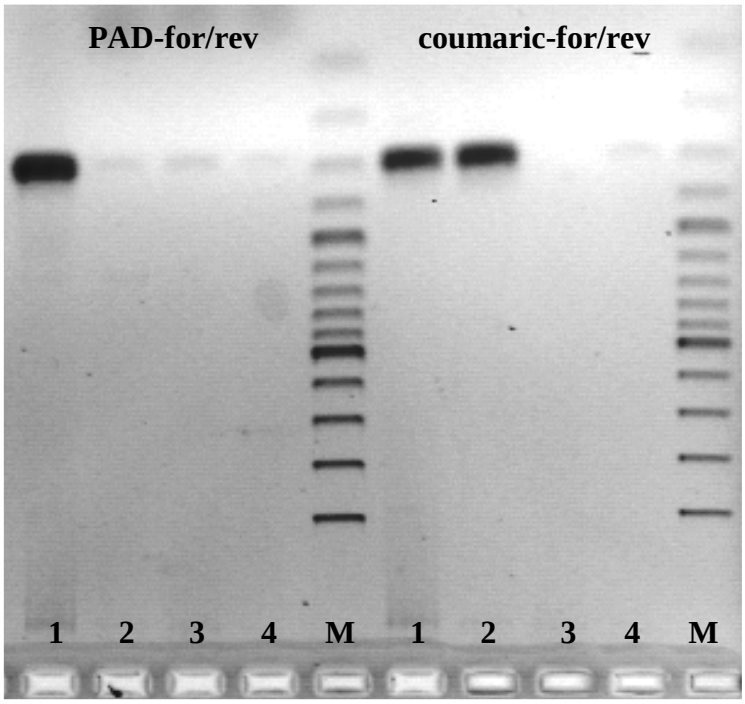

Figure 35. PCR products obtained for decarboxylase genes in lactobacilli. The used primers are also shown in table 12. Lane 1: *L. plantarum* TMW1.460, Lane 2: *L. sakei* TMW1.22, Lane 3: *L. fermentum* TMW1.890, Lane 4: *L. sanfranciscensis* TMW1.53, M: 100 bp standard.

The activity of decarboxylases in lactobacilli was evaluated in fermentation studies with mMRS supplemented with ferulic acid (FA) and coumaric acid (*p*-CA), respectively. Both substrates were tested, because of the diverse substrate preference of phenolic acid decarboxylases (46). The fermentations and subsequent analyses of supernatants were conducted as stated in chapter 3.1.9.1. Various lactobacilli were identified able to decarboxylate the hydroxycinnamates to 4-vinylguaiacol and 4-vinylphenol.

Table 19. Screening for harbored *pdc/pda* genes and enzyme activities of lactobacilli

Strain	***pdc/pda***	**Activity towards**	
		FA	***p*-CA**
L. plantarum TMW1.460	+	+*	+
L. plantarum TMW1.468	+	+*	+
L. pontis TMW1.1463	+	-	-
L. pontis TMW1.1469	+	+	+**
L. pontis TMW1.1086	+	++	++
L. brevis TMW1.1326	+	+(72h)	+

+, Decarboxylation to vinyl-derivate examined with HPLC; -, no decarboxylation detected; *Detection of hydroferulic acid; **Content of 4-vinylphenol decreased – increase of 4-ethylphenol

Predominantly, *ρ*-CA was converted by lactobacilli in the fermentation approaches. After 24 hours of fermentation, the supplemented *ρ*-CA was completely converted to 4-vinylphenol by *L. plantarum* (TMW1.460 and TMW1.468), and *L. brevis* TMW1.1326. *Lactobacillus pontis* TMW1.1086 accumulated the maximum amounts of 4-vinylphenol compared to other lactobacilli during the fermentation period. As shown in figure 36, 4-vinylphenol was accumulated to higher amounts after 48 hours in fermentations supplemented with coumaric acid as a substrate compared to the generation of 4-vinylguaiacol from ferulic acid as a substrate.

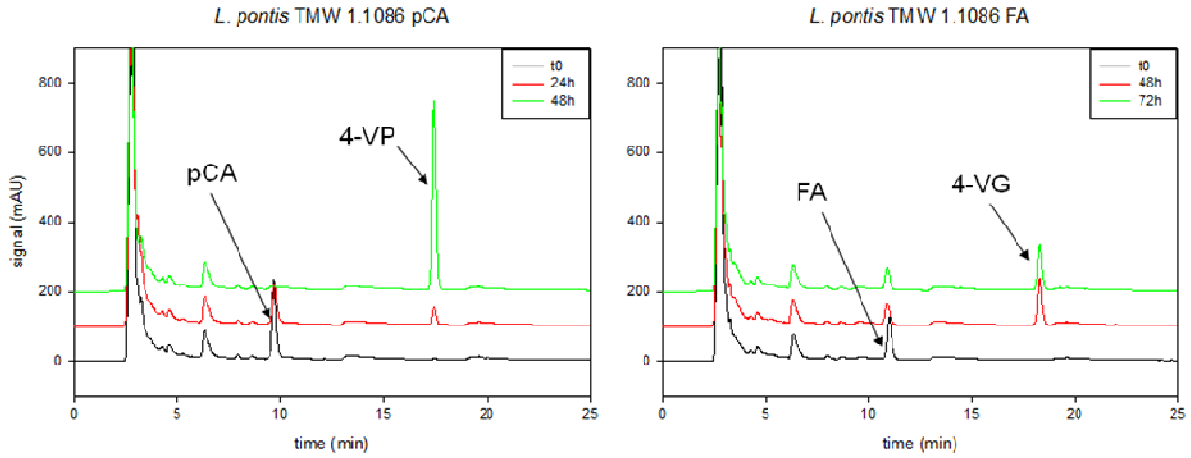

Figure 36. Metabolism of coumaric acid (left) and ferulic acid (right) in fermentations with *L. pontis* TMW1.1086. Shown are the time-points: t= 0 directly after inoculation, t= 24 h, t= 48 h, and t= 72 h.

4-vinylphenol was converted exclusively by *L. pontis* TMW1.1469 to 4-ethylphenol during the fermentation period. The strains of *Lactobacillus plantarum* metabolized the supplemented ferulic acid almost completely; however, 4-vinylguaiacol was not accumulated, but the strains reduced ferulic acid to hydroferulic acid. *L. brevis* TMW1.1326 generated only minimal amounts of 4-vinylguaiacol after 72 hours of fermentation.

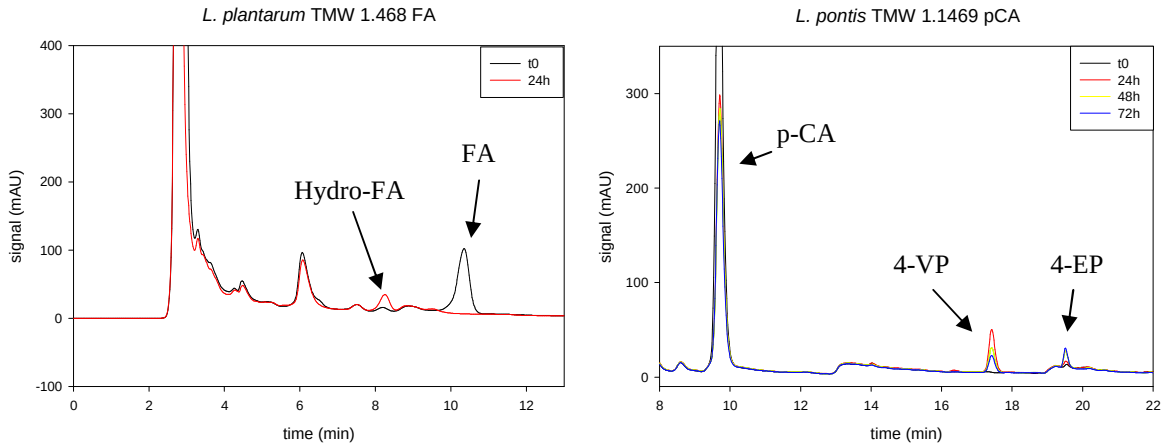

Figure 37. Metabolism of p-CA to 4-vinylphenol (highest peak after 24 hours) and 4-ethylphenol (highest peak after 72 hours) by *L. pontis* TMW1.1469 (right). Accumulation of hydroferulic acid after 24 hours of fermentation with *L. plantarum* TMW1.468 and ferulic acid as a substrate (left).

3.2.7 Wort fermentations with a combination of aroma active starter cultures

Subsequently, combinations of strains were chosen to perform wort fermentations. As stated in chapter 3.2.4 and following passages, α-L-arabinofuranosidase and xylosidase genes were detected, heterologously expressed and characterized in *L. brevis* TMW1.1326; therefore, *L. brevis* was also picked for wort fermentations. The following culture fermentation combinations were performed, and the accumulated aroma active compounds were analyzed after 120 hours:

1.) *Lactobacillus brevis* TMW1.1326
2.) *Lactobacillus brevis* TMW1.1326 and *Lactobacillus pontis* TMW1.1086
3.) *Lactobacillus brevis* TMW1.1326 and *Lactobacillus plantarum* TMW1.460
4.) *Lactobacillus pontis* TMW1.1086

10 µM free ferulic acid and 4 µM free coumaric acid were detected in the substrate (wort) at the fermentation start. Predicted cinnamoyl esterase activity in *L. brevis* TMW1.1326 (see also chapter 3.2.2) could account for liberating almost exclusively *p*-CA during wort fermentation. The chemically acidified reference did not show any liberation of *p*-CA and after 120 hours of fermentation only 2 mM ferulic acid were liberated. Thus, only 4-vinylphenol was accumulated during wort fermentations with *L. brevis* TMW1.1326. The assumption from the screening approach (see chapter 3.2.6) that the strains predominantly converted *p*-CA could also be seen in the wort fermentations. Fermentations with a culture combination of *L. brevis* TMW1.1326 and *L. pontis* TMW1.1086 increased the 4-vinylguaiacol amount as expected. The results clearly demonstrated that the predicted cinnamoyl esterase of *L. brevis* predominantly liberated coumaric acid, because an increase amount of 4-vinylphenol was accumulated in relation to the amount of free available *p*-CA. On the other hand, 4-vinylguaiacol was not accumulated to higher amounts; not the whole quantity of free ferulic acid was converted to 4-vinylguaiacol.

Table 20. Results of the wort fermentations with a combination of cultures.

Strain	TPC (24h)	pH (24h and 120h)	Metabolites (120h)
1.) *L. brevis* TMW 1.1326	5.0 x 10^8	3.9	no 4VG; FA decrease to < 2 µM
		3.5	24.5 µM 4VP; 1.8 µM*ρ*-CA
2.) *L. brevis* TMW1.1326	3.0 x 10^8	3.8	10.6 µM 4VG; 0.7 µM FA
L. pontis TMW1.1086		3.4	58.7 µM 4VP; *ρ*-CA converted
3.) *L. brevis* TMW1.1326	1.0 x 10^{10}	3.5	no 4VG; FA converted; HFA
L. plantarum TMW1.460		3.3	7.0-9.3 µM 4VP; 20.8 µM*ρ*-CA
4.) *L. pontis* TMW1.1086	2.9 x 10^8	4.1	6.9 µM 4VG; 0.7 µM FA
		3.6	56.5 µM 4VP; 0.4 µM*ρ*-CA

TPC, total plate count; FA, ferulic acid; *p*-CA, coumaric acid; 4VG, 4-vinylguiacol; 4VP, 4-vinylphenol; HFA, hydroferulic acid.

Fermentations with a culture combination of *L. brevis* TMW1.1326 and *L. plantarum* TMW1.460 accumulated hydroferulic acid instead of 4-vinylguaiacol; surprisingly, 4-vinylphenol was generated in lesser amounts as expected, in the previous screening approaches (see chapter 3.2.6) almost 100% of *ρ*-CA was converted to 4-vinylphenol. In wort fermentations with *L. pontis* TMW1.1086 almost equal amounts of 4-vinylguiacol and 4-vinylphenol were detected in comparison to the fermentation approach with the mixed fermentation culture of *L. brevis* TMW1.1326 and *L. pontis* TMW1.1086.

3.2.8 Sourdough fermentations with *Lactobacillus pontis* TMW1.1086

Three independent wheat sourdough fermentations with *L. pontis* TMW 1.1086 were performed to evaluate the accumulation of 4-vinylguaiacol during the fermentation period. An unfermented reference served as a control. The growth rates of the inoculated strain were checked after 24 hours and 48 hours of fermentation by total plate counts and RAPD-PCR. *L. pontis* TMW1.1086 exhibited average plate counts of 3.3 x 10^8 cfu/g dough after 24 hours, and 2.0 x 10^8 cfu/g dough after 48 hours. The pH-value of the dough dropped from 6.25 to a pH-value of 3.5. Dough samples were analyzed at FSI by using GC/MS; due to deficiency of an internal standard no quantification could be assured. Nonetheless, in the control dough only traces of 4-vinylguaiacol were detected. In the doughs fermented with *L. pontis* TMW1.1086 a distinct peak for 4-vinylguaiacol was observable; however, there could not be detected any clove-like aroma in the dough due to underdose concentrations of 4-vinylguaiacol beyond the aroma threshold.

3.3 DISCUSSION

3.3.1 Determination of activities in lactobacilli able to liberate free forms of hydroxycinnamates during fermentations

Phenolic acids in wheat are mostly present in bonded form through ester linkages to arabinoxylan chains (see figure 23 and 9, 26), these fiber-bond phenolic acids could be potentially released during fermentations. Different wheat cultivars showed high variability in the total phenolic contents; nonetheless, highly significant correlations between the hydroxycinnamates could be seen (25); *p*-coumaric acid and all the diferulic acid derivates were evaluated, resulted in a content estimation of the diferulic acid derivates when the ferulic acid concentration is known. Therefore, in addition to the selection of capable starter cultures in respect to gain designated aroma profiles, attention should be addressed to the right choice of wheat cultivar. Many studies focus on bioprocessing of cereal grains to improve *in vitro* bioaccessibility of phenolic compounds; mainly commercially available enzyme mixtures are used composed of xylanase, β-glucanase, α-amylase, cellulase, and also ferulic acid esterase (2, 39, 42).

In a first step, plate screenings of different lactobacilli to liberate bonded phenolic acids showed an insight into the ability of positive tested lactobacilli to act as starter cultures for generating relevant active odors in wheat sourdough fermentations. The screening approach was performed and provided similar results as previous studies (12, 19, 23). Affirmed were the results from the plate screening approach by broth fermentations with selected lactobacilli and supplemented hydroxycinnamate esters (see chapter 3.2.3). Moreover, more than 30 feruloyl esterases have been purified and characterized from fungi (49); in addition also from a wide range of bacteria and yeasts (10, 11, 20, 41). A feruloyl esterase from a metagenomic library was classified as a FEF1 family (subfamily 1B) feruloyl esterase and aligned amongst others with an acetylesterase from *Lactobacillus plantarum* WCFSI (NP_786061), showing similar sequences (10). Contradictory to these hypothetical sequence comparisons are the results of Hole et al. (26), showing that *L. plantarum* WCFSI liberated under the stated conditions only lesser amounts of free ferulic acid in comparison with other lactobacilli. In turn, the *L. plantarum* WCFSI sequence (NP_786061) showed no similarities of predicted esterases with any sequences of the 78 genomes in the genus lactobacilli (data not shown). Thus, a clear classification of predicted feruloyl esterases in the family *Lactobacillaceae* is challenging. It seems that common enzymes like hydrolases/esterases/lipases of lactobacilli

could account for a ferulic acid release during fermentations of different substrates. Their activities towards the bonded phenolic compounds could be a combination of enzymatic reactions and the existing redox potential in the system. Moreover, endogenous wheat flour enzymes could contribute to the release of ferulic acid during sourdough fermentations, the continuous pH-drop from approx. 7 to 3.5 during microbiological fermentations serves global enzymatic optima.

Based on the results of chapter 3.2.2 all heterologously expressed enzymes could be termed as cinnamoyl esterases; supportive were the results by Lai et al. (32). The characterization of the heterologously expressed cinnamoyl esterases from the selected lactobacilli showed generally a peakedness of activity between a pH-value of 7.0-8.0. This result is in agreement with a feruloyl esterase from a Holstein cow rumen library, it was shown that the pH-optimum of FAE-SH1 was around 8.0 (10). The temperature-dependent activity of the recombinant enzyme of *L. gasseri* showed an optimum at 45°C with ρ-nitrophenyl acetate as the substrate, whereas the FAE-SH1 enzyme showed a maximum of activity towards methyl ferulate at 40°C (10). A feruloyl esterase from a soil metagenomic library displayed optimal activity at 40°C and pH 6.8, the recombinant enzyme was stable in a broad pH range of 5-10 over 24 hours (49). Furthermore, a feruloyl esterase from *Streptomyces ambofaciens* was partially purified from culture supernatant; the enzyme was optimally active at pH 7.0 and 40°C (29). The purification and characterization of an extracellular feruloyl esterase from the thermophilic anaerobe *Clostridium stercorarium* was conducted by Donaghy et al. (20), the purified feruloyl esterase had an apparent mass of 33 kDa and showed optimum activity at pH 8.0 and 65°C. Besides, the purified enzyme released ferulic, ρ-coumaric, caffeic and sinapinic acid from the respective methyl esters. The recombinant enzymes in this study were also active in reactions against methyl coumarate, methyl caffeate, and ethyl ferulate, respectively (data not shown). Hence, the characterization results for the heterologous expressed cinnamoyl esterases evaluated in this study are in accordance with other approaches from different bacteria. It has to be noted, that the recombinant enzymes showed substrate side-chain length-dependent activity towards different ρ-nitrophenyl esters, indicating only less activities towards longer side chains (see chapter 3.2.2.4). Thus, the cinnamoyl esterases could be only active against short chain esters of hydroxycinnamates, e.g. ethyl ferulate, methyl coumarate. Therefore, an enzymatic liberation enforced by lactobacilli of bonded ferulic acid out of the arabinoxylan during wheat sourdough fermentations is questionable, because of the networked ferulic acid with arabinoxylan, and possible cross-linking forming

diferulates. In fermentation studies with lactobacilli in MRS broth supplemented with γ-oryzanol from rice, no liberation of free ferulic acid could be detected under the chosen conditions (data not shown). Steryl ferulates of γ-oryzanol were only hydrolyzed by cholesteryl esterase (40); lipase preparations from different sources (animal, plant, bacteria, and fungi) were not able to hydrolyze steryl ferulates of γ-oryzanol. Furthermore, cholesterol esterase was capable of hydrolyzing sitosteryl ferulate and campesteryl ferulate, which are present only in small amounts in rice γ-oryzanol but are the major steryl ferulates in other cereals such wheat and rye (38). The total steryl ferulate content of wheat grains was 6.3 mg/100 g, and the steryl ferulate content ranged from trace amounts in flours with low ash content to 34 mg/100 g in wheat brans, respectively (24, 43). As shown in figure 24, the free ferulic acid content in the experiments with flour "Tommi" was approx. 2.4 µg/ g, thus being the 100-fold minor content of ferulic acid as postulated for steryl ferulates.

The ability of lactobacilli to liberate ferulic acid in wheat sourdough fermentations was shown for *L. sanfranciscensis* TMW1.53 (see figure 24), thus the lactobacilli genomes were screened using database for arabinoxylan degrading enzymes. *L. brevis* TMW1.1326 encoded several proteins with the ability to hydrolyze glycosylated plant secondary metabolites. The characterization of the heterologous expressed α-L-arabinofuranosidase was shown in this study and from Michlmayr et al. (37). The recombinant enzyme showed optimal activity at a pH-value of 6.5 and 45°C, whereas the heterologously expressed Abf2 of *L. brevis* exhibited their optimal activity towards p-nitrophenyl-α-L-arabinofuranoside at pH 5.5 and over a temperature range of 40–60°C (37). The discrepancies could be explained with the different types of buffer used in each study. For the recombinant β-xylosidase an optimal activity could be seen at pH 7.0 and 25°C, these results are comparable to a measured activity of β-D-xylosidase by Michlmayr et al. (36). Moreover, a β-D-xylosidase from *Bifidobacterium breve* K-110 was characterized having K_m and V_{max} values towards p-nitropenyl-β-D-xylo-pyranoside of 1.45 mM and 10.75 µmol/min/mg (28), and an optimal activity at pH 6.0 and 45°C. As mentioned, the temperature optimum for the xylosidase in this study was at 25°C, indicating that the encoding gene was from a mesophilic microorganism. It was also shown, that the heterologously expressed enzymes were active in a coupled reaction against water-soluble wheat arabinoxylan. Also, the activities against branched polysaccharides derived from natural plant cell walls suggest that these enzymes may play an important role in the degradation of hemicelluloses in the diet of *L. brevis* TMW1.1326. Liberated phenolic compounds could be further metabolized and account for aroma in sourdough fermentations.

In vitro fermentations by human fecal microbiota of wheat arabinoxylan showed that a 66 kDa fraction was particularly selective for lactobacilli. Interestingly, the phenolic content from this fraction contained nearly 60% more bond ferulic acid than the 354 kDa fraction, the other way round could be seen in the bonded diferulic acid (27). Thus, larger fractions of arabinoxylan composed of diferulates were probably not as accessible for lactobacilli as the minor fractions.

In PCR screening approaches for phenolic acid decarboxylase genes in lactobacilli, several strains were determined harboring a PDA/PDC gene. Thus, the ability to metabolize hydroxycinnamic acids by lactobacilli during wheat sourdough fermentations could lead to a revised selection of starter cultures, thereby enhancing desired active aroma odors. The substrates ρ-coumaric acid and ferulic acid were decarboxylated during broth fermentations with all chosen lactobacilli (see chapter 3.2.6) to their correspondent vinyl derivate. Notwithstanding were the results for the *L. plantarum* strains TMW1.460 and TMW1.468 in fermentations with supplemented ferulic acid, both strains accumulated hydroferulic acid (see figure 37). A proposed pathway for the degradation of ρ-coumaric acid in *L. plantarum* supported this result, a knockout mutant of the ρ-coumarate decarboxylase gene showed two other inducible enzymatic activities involved in phenolic acid metabolism (4). The LPD1 mutant accumulated phloretic acid or 4-ethylphenol in fermentations with supplemented ρ-coumaric acid. Thus, a phenolic acid reductase and a putative phloretic acid decarboxylase were assumed for phenolic acid metabolism in *L. plantarum*. During the fermentation period with *L. pontis* TMW1.1469 and supplemented ρ-coumaric acid, a decrease in accumulated 4-vinylphenol was detected and an increase of 4-ethylphenol after 48 hours of fermentation (figure 36). An enzymatic background for this reaction is proposed also by other studies (4, 47, 50). The ability of *L. brevis* TMW1.1326 to degrade ferulic acid to its correspondent vinyl derivate guaiacol was not incisive, after 72 hours of fermentation only traces of 4-vinylguaiacol could be detected. Curiel et al. confirmed this outcome; moreover, *L. brevis* strains were unable to subsequently reduce or metabolize the accumulated vinyl derivates. In *L. brevis,* 4-vinylphenol, 4-vinylcatechol, and 4-vinylguaiacol were the final metabolic products from ρ-coumaric, caffeic or ferulic acids, respectively (13). *L. pontis* TMW1.1086 accumulated the highest amounts of 4-vinylphenol or 4-vinylguaiacol from the corresponding phenolic acids; however, metabolism of phenolic acids by *L. pontis* was conducted in favour of ρ-coumaric acid. In PCR screenings two putative phenolic acid decarboxylase sequences were obtained for *L. pontis* TMW1.1086. The first sequence from *L. pontis* TMW1.1086

showed highest similarities with phenolic acid decarboxylase from *L. fermentum* (AAF82764; 89% identity and 98% similarity in 81 amino acids) and *L. farciminis* (ZP_08577747; 90% identity and 96% similarity in 81 amino acids). The other putative decarboxylase sequence from *L. pontis* TMW1.1086 showed highest similarities with ρ-coumaric acid decarboxylase from *L. plantarum* JDM1 (YP_003064516; 97% identity and 97% similarity in 78 amino acids) and *Pediococcus acidilactici* DSM20284 (ZP_06196879; 88% identity and 95% similarity in 78 amino acids). In wort fermentations with a combination of aroma active starter cultures, the results from the screenings were merged together; a combination of *L. brevis* TMW1.1326 and *L. pontis* TMW1.1086 showed capable results related to the generation of active aroma odors. With the culture combination and wort as the fermentation substrate, almost 11 μM 4-vinylguaiacol and 60 μM 4-vinylphenol were accumulated after 120 hours. The combination of cultures in wort fermentations was also performed in a study of van Beek et al.; it was assumed that the combined activities of bacteria and yeast decarboxylate ρ-coumaric acid, and would then reduce 4-vinylphenol to 4-ethylphenol more effectively than either microorganism alone in pure cultures (50).

The ability to generate active aroma odors in wheat sourdough fermentations was shown for *L. pontis* TMW1.1086, a distinct peak for 4-vinylguaiacol was observable. Odor-active compounds in whole meal (WWF-F) sourdough and sourdough made from white wheat flour (WF 550-F) were evaluated; a clove-like and vanilla-like aroma were detected in higher concentrations in whole meal sourdough compared to sourdoughs prepared with white wheat flour (14). Thus, the selection of strains capable of generating aroma active substances in wheat sourdough fermentations and the best choice of wheat cultivar in respect to phenolic contents could lead to aroma enhancement. A combination of strains for substrate fermentations apparently has potential in regard to the ability to liberate improved aroma doughs; combined yeasts and lactic acid bacteria cultures were used in cereal associated fermentations since the beginning of time. The central theme of this chapter was to show that lactobacilli have the genetic background for arabinoxylan degrading enzymes; thus, liberating free forms of phenolic acids, e.g. ferulic –and coumaric acid, respectively. In addition, the redox potential in the dough system could further the liberation of bonded phenolic compounds; thus, the determination of redox potential during wheat sourdough fermentations could also help in the decision to the appropriate starter culture. The free form of phenolic acids could contribute to enhancement of aroma in wheat sourdough fermentations due to microbial decarboxylation reactions. With the multitude of sequenced *Lactobacillus* genomes

and characterizations of novel species in the genus *Lactobacillus* the alternatives for starter culture selection are increasing.

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4

4. INSIGHTS INTO THE OXIDATIVE STRESS RESPONSE OF *LACTOBACILLUS SANFRANCISCENSIS*

Lactobacillus (L.) sanfranciscensis (see also chapter 1) is an aerotolerant and obligatory heterofermentative microorganism frequently predominant in traditionally produced sourdoughs. In the early phase of evolvement, sourdough contains a lot of dissolved oxygen; it could be also introduced into the dough during the kneading. In both situations, an oxygen tolerant organism has advantages in competition as compared to strictly anaerobic organisms (26).

In this study the contribution of glutathione reductase to oxygen tolerance of *L. sanfranciscensis* TMW1.53 was examined and compared to a CyuC defective mutant of *L. reuteri.* Deletion of the cell-wall bound cystine-binding protein CyuC in *L. reuteri* decreases oxygen tolerance of the mutant strains (46). The CyuC of *L. reuteri* (previously MAP, mucus adhesion protein and BspA, basic surface protein) is part of an operon consisting of a cystathionine-γ-lyase (CgL), an ATP-binding protein, a hydrophobic membrane protein and a surface-bound cystine binding protein (46, 56). CgL accepts cysteine, cystine, and methionine as substrates for conversion to low-molecular weight thiol compounds (52). It was suggested that four proteins encoded by the operon act in concert by extracellular aggregation: ATP-dependent transport and conversion of cystathionine-γ-lyase (CgL). Furthermore, the influence of Nox-activity in *L. sanfranciscensis* TMW1.53 was evaluated under aerobic conditions to maintain the catabolic flux. By inactivation of the native NADH-oxidase gene, the hypothesis should be ensured that besides fructose O_2 can react as an electron acceptor. The effect of agitation and aeration on accumulation of organic acids during broth fermentations should show the direct impact of the NADH oxidase on regenerating pyridine nucleotides to maintain the catabolic flux. Moreover, using transport-studies, a manganese-dependent growth response should be demonstrated in *L. sanfranciscensis* TMW1.53.

An insight of the oxidative stress response in *L. sanfranciscensis* should be devised, also in respect to other enzymatic reactions involved when cells were exposed to oxidative stress.

4.1 MATERIALS AND METHODS

4.1.1 Used strains, media, plasmids, primers, and growth conditions

Lactobacillus sanfranciscensis was cultivated in MRS-media (composition per L: 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 g Tween 80, 0.2 mg each of biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamin, riboflavin, cobalamin and panthothenic acid), with slight modifications for five different MRS-compositions: MRS1: + 1 ml manganese/magnesium-stock solution (0.1 g $MgSO_4$ x $7H_2O$, 0.05 g $MnSO_4$ x H_2O per L), + 0.5 g L^{-1} cysteine, containing 10 g x L^{-1} maltose, 5 g x L^{-1} fructose and 5 g x L^{-1} glucose; MRS2: - Mn/Mg-stock solution, + 0.5 g L^{-1} cysteine, containing 10 g x L^{-1} maltose, 5 g x L^{-1} fructose and 5 g x L^{-1} glucose; MRS3: - Mn/Mg-stock solution, - 0.5 g L^{-1} cysteine, containing 10 g x L^{-1} maltose and 5 g x L^{-1} glucose; MRS4: - Mn/Mg-stock solution, + 0.5 g L^{-1} cysteine, containing 10 g x L^{-1} maltose and 5 g x L^{-1} glucose; MRS5: + Mn/Mg-stock solution, – 0.5 g L^{-1} cysteine, containing 10 g x L^{-1} maltose and 5 g x L^{-1} glucose. MRS1 was used for all the experiments concerning the glutathione reductase of *L. sanfranciscensis*; MRS1-MRS5 media were used in the approaches related to the NADH oxidase (nox) of *L. sanfranciscensis*. To maintain plasmids, 10 µg L^{-1} erythromycin was added to the nutrient where indicated. *L. sanfranciscensis* TMW1.53 was cultivated under anaerobic conditions at 30ºC, *L. reuteri* BR11 (56) and *L. pontis* TMW1.84 at 37°C. *E. coli* DH5α was cultivated aerobically in LB medium at 37 °C, for maintaining plasmids 100 µg x L^{-1} ampicillin was added. Anaerobic cultures were incubated in 50 ml falcon tubes (Sarstedt, Germany); for aerated cultures, *L. sanfranciscensis* was incubated in 300 ml shaking flasks at 30°C in an orbital shaker at 220 rpm. The plasmid pME-1 (Ehrmann et al., 2004, unpublished) was transformed in *E. coli* DH5α and was used for the construction of the integration vectors pME-1Δ*gshR* and pME-1Δ*nox* as described below. The plasmid PNG201 was transformed in *L. reuteri* BR11 according to Turner et al. (56), a L-cystine uptake system mutant was established by disruption of the *bsp*A locus.

Table 21. Used primers for PCR and cloning experiments

Primer	**Sequence (5` to 3`)**	**Use**
gshknockV	TAT ATG GAT CCA ACA TGA TGT TAA GGA AT	Cloning
gshknockR	TAT ATG GAT CCA TTC GAA AAT GGC AGT TG	Cloning
GTDHV	TAT ATT TGG GGA GTG GAC	PCR
GTDHR	ATT CGA AAA TGG CAG TTG	PCR
GTDHV1	GGG AGT GGA CAT GGA ACG	Southern Blot
GTDHR2	ATT CGA AAA TGG CAG TTG	Southern Blot
eryV	GAC TCA AAA CTT TAT TAC TTC	Cloning

T7	GTA ATA CGA CTC ACT ATA GGG C	Cloning
Deg-gshRV	GGY GGH ACT TGY CCW AAY	Screening
Deg-gshRR	ATH CCS ACT TGM GCW A	Screening
gshRV1	GTG ATC AGG CAG AAG ATT C	inverse PCR
gshRR1	GCA ATC ACA ATT TTA TCT GC	inverse PCR
gshRV2	AGA TTC AAT TAG TAC GAT TCT	inverse PCR
gshRR2	CAA TTA ATC TCT GGA ATT CCA	inverse PCR
cyuC-for3	GCT CCT TAT GCT TAT C	cDNA
cyuC-rev3	CGT GCA TCA AAT CTT TG	cDNA
nox-knock-for	TAT ACT GCA GGC AAT CTT GGA GCT AAC G	Cloning
nox-knock-rev	TAT ACT GCA GGC TAA AGG AAT GTA GGC G	Cloning
Nox-for1	GCA ATC TTG GAG CTA ACG	Southern Blot
Nox-rev1	GCT AAA GGA ATG TAG GCG	Southern Blot
Nox-klon-for	TAT ACC ATG GTT ATG AAA GTT ATT GTA GTA	Cloning
Nox-klon-rev	TAT ATC TAG ATA TTT ATG TGC TTT GTC AGC	Cloning
MntH1-for	GCA CCA TAA ACT AAT TGA	cDNA
MntH1-rev	GAC TTA CTA GTT CTA GCC	cDNA
MntH2-for	ATG GCC GCA AAT TTA ACT	cDNA
MntH2-rev	GAA GTT GCC CTA TCT AAC	cDNA

restriction sites are underlined

4.1.2 General molecular techniques

General techniques regarding cloning, DNA manipulations and agarose gel electrophoresis were performed as described by Sambrook et al. (49) Chromosomal DNA of *L. sanfranciscensis* was isolated according to the method of Lewingston et al. (31) and *E. coli* plasmid DNA was isolated with the PegGold plasmid miniprep kit I from Peqlab GmbH (Erlangen, Germany) or with the Wizard Plus SV Minipreps DNA Purification System from Promega (Madison, USA) according to the manufacturer's instructions. Restriction endonuclease digestions and ligations with T4-DNA ligase were performed following the recommendations of the suppliers (Fermentas, St. Leon-Rot, Germany). PCR was carried out in thermocyclers (Primus 96^{plus}, MWG-Biotech, Ebersberg, Germany; Applied Biosystems, Foster City, USA) by using Arrow-Taq Polymerase and dNTP's from MP Biomedicals (Heidelberg, Germany) or Invitrogen (Burlington, Canada). PCR-products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany; Mississauga, Canada) according to the instructions of the supplier. Sequencing was carried out by the didoexy method of Sanger et al. (50) by using GenomeLab^{TM} DTCS-Quick Start Kit (Beckman Coulter, Fullerton, USA) in combination with an Applied Biosystems model 377A automated sequencing system or by the external laboratory GATC. Nucleotide and amino acid sequence analysis was carried out using the DNAsis Max for Windows software (Hitachi, Berlin, Germany) or by using the DNAStar for Windows software (DNASTAR, Madison, USA).

Transformations were performed with a Bio-Rad gene pulser apparatus (Bio-Rad Laboratories, Hercules, USA) in 0.2 mm cuvettes (Sarstedt, Nürnbrecht, Germany; Bio-Rad Laboratories, Hercules, USA) at 2.5 kV, 25 µF and 200 Ω for *E. coli* and at 1.2 kV, 25µF and 1000 Ω for *Lactobacillus sanfranciscensis* and *L. reuteri* BR11.

4.1.3 Sequence and mRNA isolation of genes coding for manganese transport protein in *L. sanfranciscensis* TMW1.53.

Genes coding for a manganese transport protein were sequenced based on a clone library of *L. sanfranciscensis* TMW1.53 done previously in our lab. RNA was isolated from cells of *L. sanfranciscensis* grown aerobically (shaking at 220 rpm) in 50 ml of MRS1 broth to an optical density of 0.5 at 590 nm. Bacterial cells in the supernatant were harvested by centrifugation (15 min, 4.500 rpm) 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 incubation with RQ1 RNase-free DNase (Promega, Mannheim, Germany). Reverse transcription was performed by incubation of RNA with random hexamer primers (Random hexadeoxynucleotides, Promega) at 70°C for 10 minutes. After cooling on ice, 1 µL dNTPs (25 mM), 1 µL reverse transcriptase (200 U μL^{-1}, M-MLV-RT, RNase H minus, Promega), 5 µL 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 the reaction was stopped by heating the sample at 72°C for 15 minutes. A fragment of the manganese transport proteins of *L. sanfranciscensis* was amplified using *Taq* polymerase and primers MntH1-for and MntH1-rev, Mnth2-for and MntH2-rev, respectively, and cDNA as template (Table 21). All PCR reactions were also carried out with DNAse-digested RNA preparations to verify absence of chromosomal DNA.

4.1.3.1 Measurement of the intracellular manganese levels in growing cells of L. sanfranciscensis using fluorescence analysis

Cells of *L. sanfranciscensis* were grown to an optical density of 1 at 590 nm under static, anaerobic conditions or shaking (220 rpm) in MRS1 or MRS2, respectively. The cells were harvested by centrifugation at room temperature (7000 x g, 15 min) and washed three times with MES buffer (0.1 M). Cells were resuspended in 0.1 M MES buffer to an optical density of 1 at 590 nm. In order to liberate free intracellular manganese (25) from the cells, a volume

of 1 ml of the cell suspension was boiled for 10 minutes in a water bath and centrifuged at room temperature (7.000 x g, 10 minutes). The supernatant was taken for the calcein fluorescence assay. Calcein emits fluorescence in the presence of metal ions such as Mg^{2+} in basic conditions; bond to Mn^{2+}, the calcein fluorescence is quenched. For determination of free manganese, 0.5 ml of supernatant was mixed with an equal amount of Tris buffer (0.5 M, pH 8.8). To ensure reducing conditions (17) 1 mM DTT was added. 20 µl of calcein solution (5 µM calcein, 16 mM $MgCL_2$ in 25 mM tris buffer pH 8.8) were added to the sample, and the fluorescence (λ_{Ex}: 488 nm, λ_{Em}: 511 nm, slit: 5 nm) was measured in a Luminescence Spectrometer LS 50B (Perkin Elmer, Rodgau-Jügesheim, Germany). For manganese quantification, a calibration curve was recorded in the range from 0.1 µM to 10 µM Mn^{2+}. The amount of intracellular manganese, which was released from the cells (at OD_{590nm}= 1) to a volume of 1 ml MES buffer, was calculated.

4.1.4 Southern hybridization

Genomic DNA was digested with *Eco*RV, *Hind*III and *Nco*I, separated on a 0.7% agarose gel, and then transferred to nylon membranes (Amersham Biosciences). A 921 bp fragment of *L. sanfranciscensis* TMW1.53 obtained with primers GTDHV1 and GTDHR2 (Table 21) was labeled with DIG-DNA Labeling Mixture (Roche Diagnostics). Hybridization and washing were performed basically according to the manufacturer's instructions. The same approach was conducted for the evaluation related to the NADH oxidase of *L. sanfranciscensis*; however, genomic DNA was digested with *Eco*RI, *Hind*III and *BamH*I. A 694 bp fragment of *L. sanfranciscensis* was obtained with primers Nox-for1 and Nox-rev1 (Table 21).

4.1.5 Creation of knock-out mutants for NADH oxidase and glutathione reductase from *L. sanfranciscensis* TMW1.53

4.1.5.1 Insertional inactivation of the NADH-oxidase gene (nox) by single crossover integration

Based on the submitted DNA sequence (Accession No.: BAB19268) of the NADH-oxidase gene from *L. sanfranciscensis* TMW1.53, primers nox-knock-for and nox-knock-rev were designed for PCR, carrying *Pst*I restriction sites (Table 21). For insertional inactivation of the NADH-oxidase gene, a 694-bp fragment of the NADH-oxidase gene was obtained with PCR using primers nox-knock-for and nox-knock-rev. Digestion and ligation into the *Pst*I restriction site of plasmid pME-1 resulted in the non-replicating integration vector pME-1Δ*nox*, which was cloned in *E. coli* DH5α and isolated with the PegGold plasmid miniprep

kit I. For preparation of electro competent cells of *L. sanfranciscensis*, the strain was grown in MRS1 broth supplemented with 1% (w/v) glycine to an optical density (590 nm) of 0.7. The cells were harvested by centrifugation at 4 °C (4000 x g, 15 minutes) and washed four times with 50 mL of 10 mM $MgCl_2$ solution, once with glycerol (10% v/v) and once with glycerol-sucrose solution (10% v/v, 0.5 M). Cells were resuspended in glycerol-sucrose solution and stored at –80°C in 100 µL aliquots. All washing and storage solutions were cooled on ice. After electroporation, cells were incubated in MRS1 at 30°C for 3 hours prior to plating on MRS1 supplemented with 10 ppm erythromycin. To verify the insertion of plasmid pME-1Δ*nox* into the NADH-oxidase gene in cells from erythromycin-resistant colonies, PCR was carried out with primers targeting the regions upstream and downstream of the NADH-oxidase gene (nox-klon-for and nox-klon-rev, respectively) and the plasmid borne regions from pME-1 (eryV, and T7, Table 21). PCR products obtained with primers nox-klon-for/eryV and nox-klon-rev/T7 were sequenced.

4.1.5.2 Insertional inactivation of the glutathione reductase gene by single crossover integration

Based on a 771 bp fragment of the glutathione reductase gene from *L. sanfranciscensis* TMW1.53 (Vermeulen et al., 2006, unpublished) primers gshRV1/V2 and gshRR1/R2 were designed for inverse PCR (Table 21). Chromosomal DNA of *L. sanfranciscensis* was digested with *Pst*I, religated, and used as template for inverse PCR to yield a product with a size of about 2.500 bp. For insertional inactivation of the glutathione reductase gene, a 765-bp fragment of the glutathione reductase gene was obtained with PCR using primers gshknockV and gshknockR (Table 21), carrying *Bam*HI restriction sites. Digestion and ligation into the *Bam*HI restriction site of plasmid pME-1 resulted in the non-replicating integration vector pME-1Δ*gshR*, which was cloned in *E. coli* DH5α and isolated with the Wizard Plus SV Minipreps DNA Purification System as recommended by the supplier. The preparation of electro competent *L. sanfranciscensis* cells, and the electroporation was performed according to chapter 4.1.5.1. PCR with primers targeting the regions upstream and downstream of the glutathione reductase gene (GTDHV and GTDHR, respectively) and the plasmid-borne regions from pME-1 (eryV and T7, Table 21) were used to verify the insertion of plasmid pME-1Δ*gshR* into the glutathione reductase gene. To ensure a single crossover integration of pME-1Δ*gshR* into the *gshR* gene of *L. sanfranciscensis* TMW1.53Δ*gshR*, the PCR products obtained with primers T7/GTDHV and GTDHR/eryV were sequenced.

4.1.6 Preparation of crude cellular extracts and determination of NADH-oxidase, glutathione reductase or cystathionine-γ-lyase activity

L. sanfranciscensis cells grown under anaerobic and aerobic conditions to mid-exponential phase were harvested by centrifugation and after disruption the crude cellular extract was used for the assay. NADH-oxidase activity in crude cellular extract was determined at 340 nm (absorption coefficient 6.22 x 10^3 M^{-1} cm^{-1}) using 0.2 mM NADH, 50 mM HEPES-buffer (pH 7.0), 1 mM dithiothreitol, and limiting amounts of the crude cellular extract. The assay was carried out at 30°C; 1 U corresponds to the oxidation of 1 µmol NADH per min. Protein concentrations were measured by the method described by Bradford (4).

GshR activity in extracts was measured at 25ºC by monitoring the oxidation of NADPH in the reaction mixture (1 ml) at 340 nm. The reaction mixture contained 640 µl sodium phosphate buffer (143 mM NaH_2PO_4, 143 mM Na_2HPO_4, mixture 1:1 + 5 mM EDTA), 120 µl GSSG (10mM), 100 µl NADPH (1mM) and 100 µl extracts. The reduced GSH-content was measured by adding 20 µl of reagent B (39.6 mg 5,5-dithiobis-(2-nitrobenzoic acid) in 10 ml 0.5 M sodium phosphate buffer, pH 7.0). After incubation for 30 min in the dark, the absorbance was measured at 405 nm. Cystathionine-γ-lyase activity was determined according to Smacchi et al. (52). Cystathionine (5 mM, final concentration) was used as substrate in the presence of 50 mM KPi buffer (pH 8.0)-PLP. The enzyme activity was determined by quantification of ketobutyrate and ammonia produced in the enzymatic reaction.

4.1.7 Determination of intracellular and extracellular sulfhydryl levels

Cystein transport by *L. sanfranciscensis* was determined according to Turner et al. (56). Cells grown aerobically to mid-exponential phase were harvested by centrifugation, washed twice in KPM solution (0.1 M K_2HPO_4 adjusted to pH 6.5 with H_3PO_4 and containing 10 mM $MgSO_4$ x 7 H_2O), and suspended in KPM to a OD of 0.5. Portions (0.5 ml each) of this suspension were supplemented with 10 µl of 10 µM L-cysteine and 10 µl of 1 M D-glucose, and the suspension was then incubated at 30°C for 1 h. The cells were then pelleted, and the supernatant was removed and put on ice. Fifty microliters of a 10 mM solution of DTNB in KPM was added to the supernatant, and the absorbance at 412 nm was measured. The pelleted cells were washed twice with 1 ml of KPM and resuspended in a solution containing 200 µl of water, 4 µl of 0.5 M EDTA, 10 µl of 1 M Tris-HCL (pH 8), 20 µl of 10 mM DTNB, and 100 µl of 10% SDS, added successively. This mixture was incubated at 30°C for one hour, cellular debris was removed by centrifugation and the absorbance of the supernatant was measured at

412 nm. Assays containing no bacterial cells or no L-cysteine served as control. Sulfhydryl concentrations were calculated based on the absorbance at 412 nm and the molar extinction coefficient of 5-thio-2-nitrobenzoic acid of 13.6 L $(mol\ cm)^{-1}$, and were corrected to an optical density of 1.0. The intracellular accumulation of thiols during incubation was calculated as follows:

$([thiol]_{(cells\ in\ KPM\text{-}cysteine)} - [thiol]_{(cells\ in\ KPM\ without\ cysteine)}) \times (cell\ density)^{-1}$

The decrease in extracellular thiols during incubation was calculated as follows:

$[thiol]_{(KPM\text{-}cysteine)} - [thiol]_{(cells\ in\ KPM\ -\ cysteine)}$

4.1.8 Sequence and expression of a gene coding for a CyuC-like protein in *L. sanfranciscensis* TMW1.53

A gene coding for a CyuC-like protein was sequenced based on several rounds of PCR with primers derived from the *cyuC* of *L. reuteri* BR11. RNA was isolated from cells of *L. sanfranciscensis* that were grown aerobically (shaking at 220 rpm) in 50 ml of MRS-broth to an optical density at 595 nm of 0.5. Bacterial cells in the supernatant were harvested by centrifugation (15 min, 4500 rpm) 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 incubation with RQ1 RNase-free DNase (Promega, Mannheim, Germany). Reverse transcription was performed by incubation of RNA with random hexamer primers (Random hexadeoxynucleotides, Promega) at 70°C for 10 minutes. After cooling on ice, 1 µL dNTPs (25 mM), 1 µL reverse transcriptase (200 U μL^{-1}, M-MLV-RT, RNase H minus, Promega), 5 µL 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 the reaction was stopped by heating the sample at 72°C for 15 minutes. A fragment of the CyuC-like protein of *L. sanfranciscensis* was amplified using *Taq* polymerase and primers cyuC-for3 and cyuC-rev3 and cDNA as template (Table 21). All PCR reactions were also carried out with DNAse-digested RNA preparations to verify absence of chromosomal DNA.

4.1.9 Determination of the optical density, pH, organic acids formed during fermentation

Samples of 250 µl were taken for determination of optical density at 590 nm (OD_{590nm}) using a plate absorbance reader (Sunrise, Tecan, Germany). Samples of 1.5 ml were taken for determination of metabolites during fermentation. The concentration of the fermentation

substrates and end products were determined by HPLC. Cells were removed by centrifugation (14,000 x *g* for 5 minutes). The concentrations of lactic acid and acetic acid in the supernatant were determined with a polyspher OAKC column (Merck, Darmstadt, Germany). The flow rate was 0.4 ml min^{-1}, the mobile phase consisted of 5 mM of H_2SO_4 $liter^{-1}$, and the temperature of the column was 70°C. The pH was measured with a glass electrode and cell counts were determined on MRS1-agar. The appropriate dilution was plated by using a spiral plater (IUL, Königswinter, Germany), and plates were incubated at 30°C for 48 hours under a controlled atmosphere (76% N_2, 20% CO_2, 4% O_2).

4.1.10 Influence of oxidants treatment on growth inhibition determined with a plate assay

Different MRS-plates compositions (according to media and growth conditions) were covered with 1 ml of an overnight culture from *L. sanfranciscensis* or the cognate mutant TMW1.53Δ*nox*. After the plates dried up, sterile sensi-discs (BD Diagnostics, Germany) supplemented with diamide (final concentration of 1 M), H_2O_2 (final concentration of 5 mM) and paraquat (final concentration of 5 M) were placed in the middle of the agar-plates. Plates were incubated at 30°C for 48 h under a controlled atmosphere (76% N_2, 20% CO_2, 4% O_2). The growth inhibition towards the oxidants was measured as the diameter of growth inhibition expressed in millimeters.

4.1.11 Determination of the effect of oxidants treatment on growth rates in MRS broth

According to Turner et al. (56) 500 µL of log-phase cells were added to 4.5 ml of MRS supplemented with 0.5 g L^{-1} cysteine or without added cysteine containing either 500 µl of sterile double-distilled water (ddH_2O) (0 mM paraquat), 166 µl of 1 M paraquat (methyl viologen; Sigma), and 333 µl of sterile ddH_2O (30 mM paraquat) or 500 µl of 1 M paraquat (90 mM paraquat). The cultures were incubated at 30°C without shaking. Growth was monitored by measuring the optical density at 590 nm (OD_{590nm}).

The influence of diamide treatment on growth rates was determined in 10 ml of MRS1 and MRS2 broth, respectively, and inoculated with overnight cultures of *Lactobacillus sanfranciscensis* and TMW1.53Δ*nox*. The strain cultures were incubated at 30°C under static conditions in both media, and were supplemented with 5 mM of diamide during a five hour time course after they reached an optical density at 590 nm of 0.4.

4.1.12 Nucleotide accession numbers

The nucleotide sequence of the *Lactobacillus sanfranciscensis* TMW1.53 glutathione reductase (*gshR*) and CyuC-like protein genes have been assigned the GeneBank accession numbers DQ866807 and EF422159, respectively. The sequence of the genomic region of nox has been registered with the DDBJ/EMBL/GenBank databases under the accession number AB035801. The nucleotide sequences of the *Lactobacillus sanfranciscensis* TMW1.53 predicted Mn^{2+}/Fe^{2+} transporters of the Nramp family MntH1 and MntH2 protein genes have been assigned the Gene Bank accession numbers FJ440569 and FJ440570, respectively.

4.2 RESULTS

4.2.1 Nucleotide sequence analysis of the *L. sanfranciscensis* TMW1.53 glutathione reductase gene

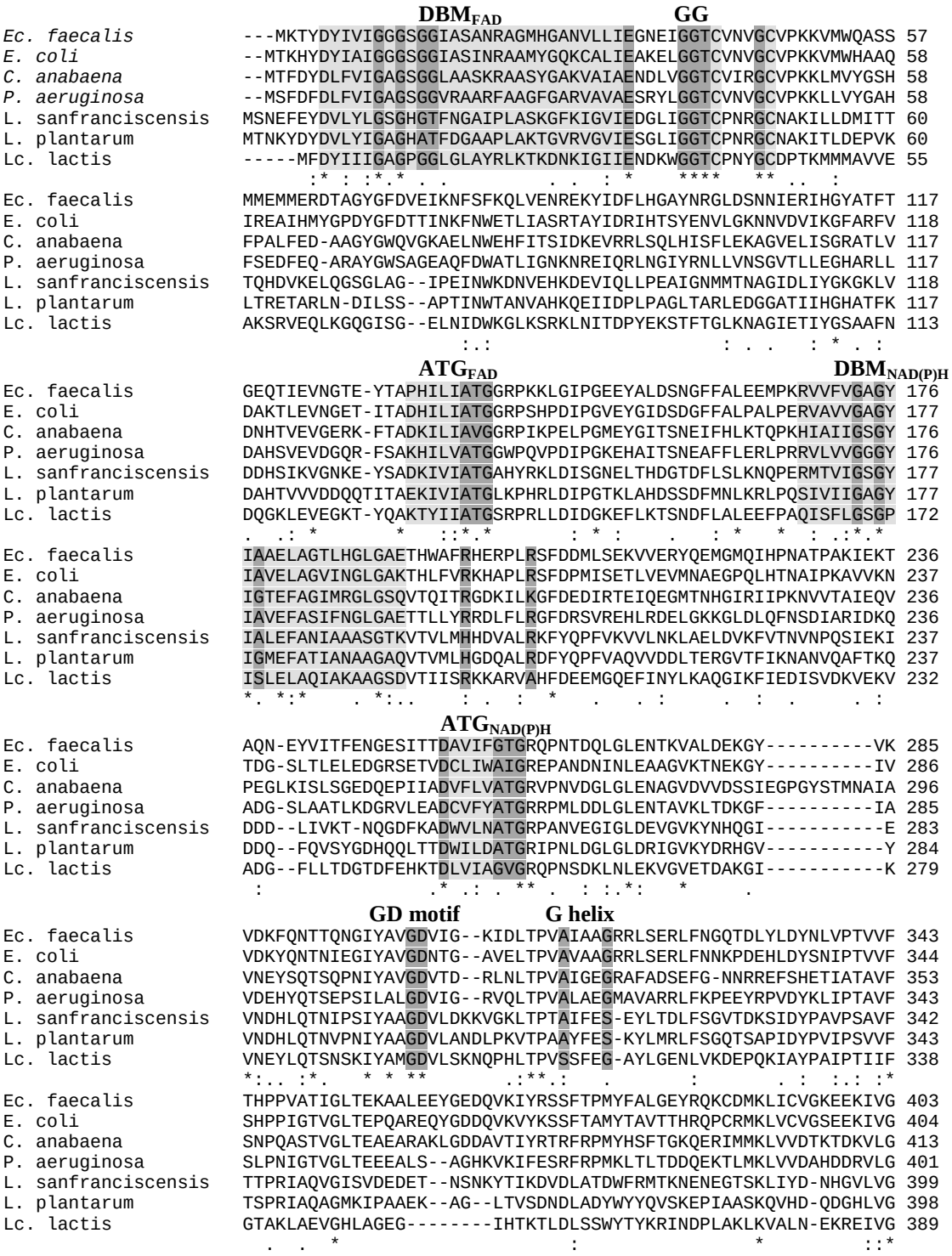
The sequence of a complete open reading frame termed *gshR* encoding a putative glutathione reductase was obtained by inverse PCR. Sequence analysis indicated the presence of an imperfect Shine-Dalgarno sequence (AAGGAG), putative -10 and -35 sequences corresponding to consensus sequences proposed for lactobacilli (38), and a palindromic sequence downstream from the termination codon (TAAAAACA-TGTTTTTA), indicating that the glutathione reductase gene is expressed as monocistronic mRNA. Southern hybridization of genomic DNA from *L. sanfranciscensis* TMW1.53 with a probe targeting *gshR* demonstrated that its chromosome harbours a single copy of the gene (data not shown).

4.2.2 Analysis of the amino acid sequence compared with bacterial glutathione reductases

GshR codes for a 446-amino-acid protein GshR with a predicted relative molecular weight M_R of 48614 and a predicted pI of 4.79 (see chapter 2.2.7, figure 18). Blast searches showed highest similarities with putative glutathione reductases from *Lactobacillus plantarum gshR2* (EMBL AL935255; 51% identity and 69% similarity in 445 amino acids) and *Lactococcus lactis* (EMBL AE006318; 33% identity and 54% similarity in 437 amino acids). Moreover, GshR exhibited high similarities to the glutathione reductases from *Cyanobacterium anabaena* (EMBL X89712; 31% identity and 58% similarity in 427 amino acids), *Pseudomonas aeruginosa* (EMBL X54201; 31% identity and 50% similarity in 444 amino acids) and *Enterococcus faecalis* (EMBL AE016830; 29% identity and 55% similarity in 436 amino acids), these enzymes were characterized on biochemical level. The glutathione reductase of *E. coli* (EMBL M13141) showed 26% identity and 53% similarity in 436 amino acids.

The superfamily flavoprotein pyridine nucleotide reductases share similarities in sequence and structure (57). The sequences of the glutathione reductases contain two dinucleotide-binding motifs (DBM), the first domain is involved in binding FAD, the second one in NAD(P)H. The GG doublet is perfectly conserved within the different glutathione reductases and appears 5 residues downstream of the DBM. This motif contains also the two redox-active cysteine-residues (see figure 38). GshR of *L. sanfranciscensis* contains two

dinucleotide-binding motifs (DBM) and the GG doublet that are highly conserved in different glutathione reductases (57). Most glutathione reductases contain the highly conserved NAD(P)H binding site sequence (GxGYIAx18Rx5R) (34); however, in *L. sanfranciscensis* the first arginine residue in the Rx5R motif is replaced by histidine. The ATG and GD motifs that are present in most flavoproteins with two dinucleotide-binding domains (57) are also present in GshR of *L. sanfranciscensis*.



```
Ec. faecalis          LHGIGIGVDEMLQGFAVAIKMGATKADFDNTVAIHPTGSEEFVTMR---- 449
E. coli               IHGIGFGMDEMLQGFAVALKMGATKKDFDNTVAIHPTAAEEFVTMR---- 450
C. anabaena           AHMVGENAAEIIQGVAIAVKMGATKKDFDATVGIHPSSAEEFVTMR---- 459
P. aeruginosa         CHMVGAEAGEILQGIAVAMKAGATKQAFDETIGIHPTAAEEFVTLRTPTR 451
L. sanfranciscensis   ATEVSDQAEDSISTILPAIEYQLTPKQIKHMISLFPTIGSESWSKL---- 445
L. plantarum          VTEISDQAEDAVNALLPAIEYQLDREQIDRLIGIFPTIGYAAWHRA---- 444
Lc. lactis            ASTVSSVADEVINLINILIQQKMTLADVEKMIFTYPTVASDLEYFY---- 435
                         :.    : :. .   ::       ..  :  .*: .
```

Figure 38. Amino acid sequence of the glutathione reductase of *L. sanfranciscensis* TMW1.53 and multiple alignment with the glutathione dehydrogenases of *Enterococcus faecalis*, *Cyanobacterium anabaena*, *Pseudomonas aeruginosa* and *Escherichia coli* as well as the putative glutathione reductases from *Lactobacillus plantarum* and *Lactococcus lactis*. The shaded boxes indicate the highly conserved dinucleotide binding motifs (DBM) for FAD-binding and NAD(P)H-binding. Other conserved sequence motifs in flavoproteins such as GC, ATG_{FAD}, GD and the G helix are also indicated (57).

4.2.3 Insertional inactivation of the glutathione reductase gene and glutathione reductase activity of the mutant strain

L. sanfranciscensis TMW1.53 was transformed with the non-replicating plasmid pME-1Δ*gshR* yielding strain *L. sanfranciscensis* TMW1.53Δ*gshR*. Sequencing of the disrupted glutathione reductase gene ensured that a single crossover integration of pME-1Δ*gshR* into the chromosomal *gshR* gene of TMW1.53Δ*gshR* has taken place (see figure 39).

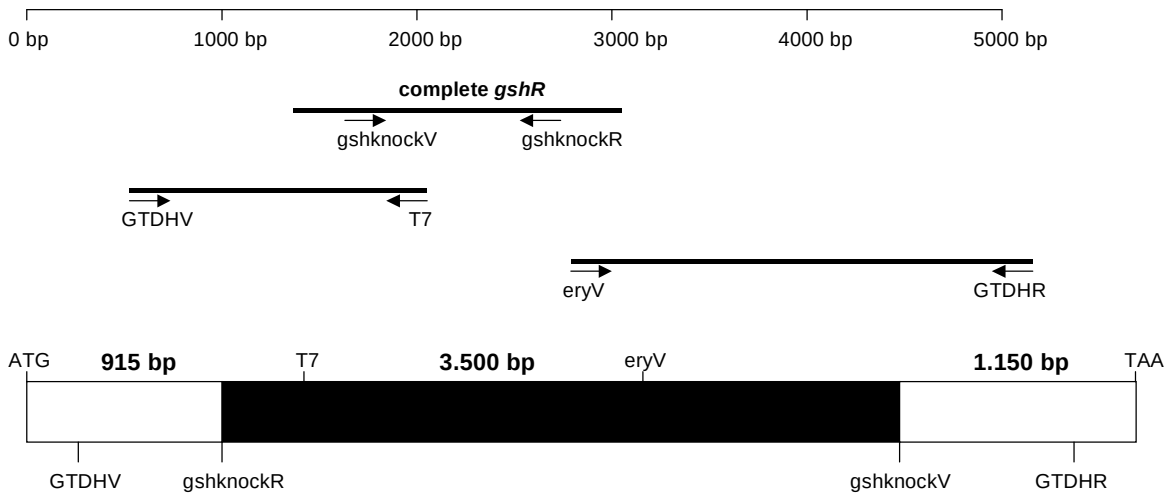


Figure 39. Integration of pME-1Δ*gshR* into *gshR* of *L. sanfranciscensis* TMW1.53. The location of primers and the corresponding PCR products that were used for sequencing of the disrupted *gshR* are indicated. Sequences derived from the integration plasmid are shaded black. ATG, start codon of *gshR*, TAA, stop codon of *gshR*.

Crude cellular extracts of the Δ*gshR*-mutant exhibited a glutathione-reductase activity of 14 nmol min^{-1} mg^{-1} compared to an activity of 45 nmol min^{-1} mg^{-1} in the wild type strain, indicating that *gshR* encodes an active glutathione reductase.

4.2.4 Contribution of the glutathione reductase to the oxygen tolerance of *L. sanfranciscensis*

The tolerance of *L. sanfranciscensis* TMW1.53 and its Δ*gshR* mutant towards oxygen and superoxide radicals was determined in mMRS medium, which contains 0.5 g L^{-1} cysteine, and in mMRS without cysteine. The wild type strain tolerated aerobic conditions in either medium whereas Δ*gshR* grew significantly slower in the presence of oxygen.

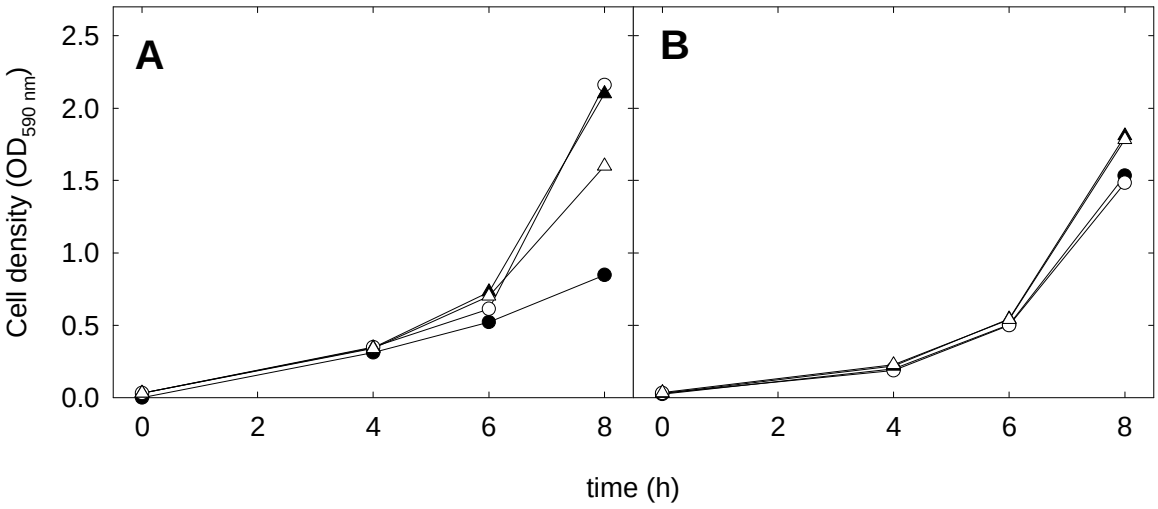


Figure 40. Growth of *L. sanfranciscensis* TMW1.53 (▲, Δ) and *L. sanfranciscensis* TMW1.53Δ*gshR* (●, ○) at aerobic conditions (black symbols) and anaerobic conditions (open symbols). Experiments were carried out in mMRS without cysteine (panel A) and in mMRS containing 0.5 g L^{-1} cysteine (panel B). Results are representative for three independent experiments.

This difference was not observed in the absence of oxygen or in the presence of 0.5 g L^{-1} cysteine (see figure 40), indicating that the *gshR* mutant strain is more sensitive to oxidative stress because it is unable to maintain a high intracellular levels of thiols. To determine the sensitivity of *L. sanfranciscensis* TMW1.53Δ*gshR* to the superoxide radicals, the growth of the mutant strain and *L. sanfransicensis* TMW1.53 were compared in growth medium supplemented with the superoxide-generating paraquat in concentrations ranging from 0 mmol to 49 mmol. Growth of *L. sanfranciscensis* TMW1.53 was unaffected by paraquat, but paraquat strongly inhibited the growth of *L. sanfranciscensis* TMW1.53Δ*gshR* in the absence of cysteine (Figure 41). When cysteine was added to the medium, paraquat did not affect growth of either strain (data not shown).

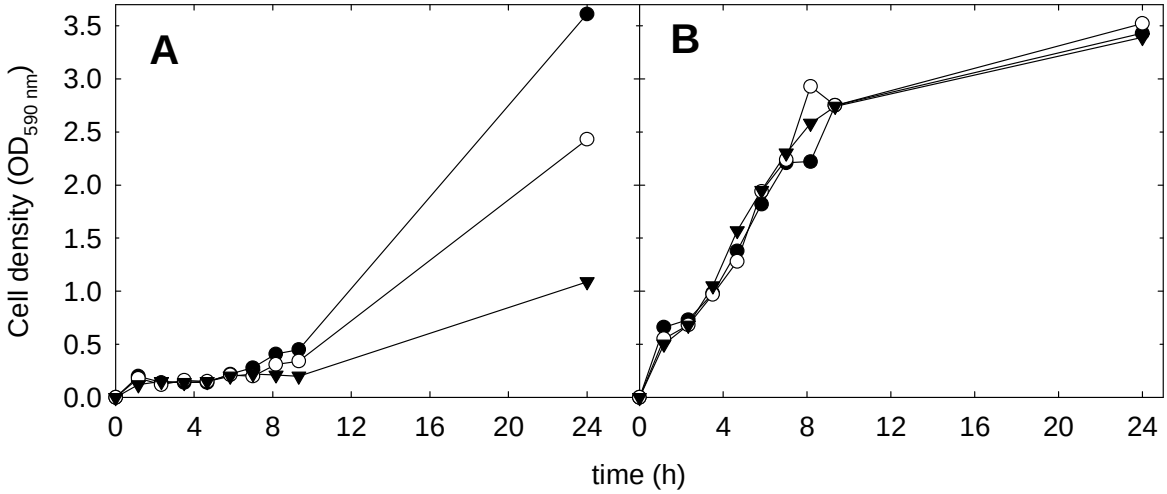


Figure 41. Growth of *L. sanfranciscensis* TMW1.53Δ*gshR* (Panel A) and *L. sanfranciscensis* TMW1.53 (Panel B) in mMRS without addition of cysteine. To the media were added 0 (●), 30 (○) or 49 (▼) mM paraquat. Results are representative for two independent experiments.

4.2.5 Cysteine and cystine transport by *L. sanfranciscensis* TMW1.53

To affirm that cysteine transport complements the protective effect of glutathione reductase during aerobic growth, cysteine transport by aerobically grown cells of *L. sanfranciscensis* TMW1.53 and TMW1.53Δ*gshR* was estimated by determination of intra- and extracellular thiol levels after incubation in buffer with cysteine. The intracellular sulfhydryl levels prior to cystein supplementation were 2.7 ± 0.8 and 2.9 ± 0.9 nmol / unit cell density for the wild type and mutant strains, respectively, and intracellular thiol levels increased by 27 ± 5 and 24 ± 6 nmol / unit cell density, respectively, upon addition of cysteine to cellular suspensions. A concomitant decrease of thiol levels in the buffer by 110 ± 7 and 103 ± 6 nmol, respectively, was observed.

L. reuteri BR11 harbors separate transport systems for cystine and cysteine. Cystin transport is mediated by CyuC and cognate ATP-binding and membrane-spanning proteins (20, 21). *L. sanfranciscensis* TMW1.53 harbors an open reading frame coding for a 264 amino acid protein. The predicted gene product is 46% identical 63% similar to *cyuC of L. reuteri* BR11. The expression of *cyuC* in *L. sanfranciscensis* was verified by amplification of a 637 bp fragment of *cyuC* from cDNA library obtained from exponentially growing cells of *L. sanfranciscensis* TMW1.53. Moreover, the genome sequence of *L. sanfranciscensis* TMW1.1304 is harboring a permease for L-cystine transport *tcyB* (60).

4.2.6 Gene arrangement and regulation of *nox*

The nox gene is of 1356 bp length and translation renderes a protein of 452 aa length, with 67% homology to the NOX of *L. brevis* (15). Putative -10 and -35 promoter sequences were found as TACAAT and as TTGTGT located -41 to -52 and -70 to -75 nt upstream the *nox* start codon. A palindromic sequence located downstream of *nox* could function as a termination signal, covered by nt 1543-1559 and nt 1569-1587 (Accession AB035801). Taken together, these observations indicate that the nox gene of *L. sanfranciscensis* is transcribed as monocistronic unit. A sequence TGTAAACGATTA-CA spanning from nt -27 to nt -40 from the *nox* start codon, showed high homology to a variety of *cre* sequences proven or believed to be involved in catabolite regulation in Gram-positive bacteria. The positional distribution of cre´s known to be active has been reported to be within approximately +/- 200 bp of the translation start site (19). In agreement with this, the *nox cre* sequence is located between the-10 region and the probable ribosome binding site.

4.2.7 Insertional inactivation of the NADH oxidase gene and Nox activity of the mutant strain

L. sanfranciscensis TMW1.53 was transformed with the non-replicating plasmid pME-1Δ*nox* yielding strain *L. sanfranciscensis* TMW1.53Δ*nox*. Sequencing of the disrupted NADH oxidase gene ensured that a single crossover integration of pME-1Δ*nox* into the chromosomal *nox* gene of TMW1.53Δ*nox* has taken place. Moreover, by southern hybridization the integration success of the non-replicating plasmid PME-1Δ*nox* into the native NADH oxidase gene was assured. Crude cellular extracts of the Δ*nox*-mutant exhibited a specific NADH oxidase activity of 0.01 U mg^{-1} compared to an activity of 0.10 U mg^{-1} in the wild type strain, indicating that *nox* encodes an active NADH oxidase. The growth condition had no effect on specific NADH-oxidase activity; activities were the same under aerobic and anaerobic conditions in *L. sanfranciscensis* TMW1.53 cultures grown in MRS1.

4.2.8 The effect of aeration on growth in different media compositions

The growth of *Lactobacillus sanfranciscensis* TMW1.53 and its cognate mutant TMW1.53Δ*nox* in different MRS media compositions were determined in static, anaerobic conditions and compared with the growth velocity in aerated cultures (see figure 42). Under static, anaerobic growth conditions the wild type strain (panel A) and the mutant strain (panel C) exhibited growth only in MRS media supplemented with fructose. The final OD after 24

hours was higher and the growth velocity was increased in cultures of the wild type strain compared to the mutant strain in MRS2. In both strains, the lag-phase was highly increased in media without fructose supplementation during the time course.

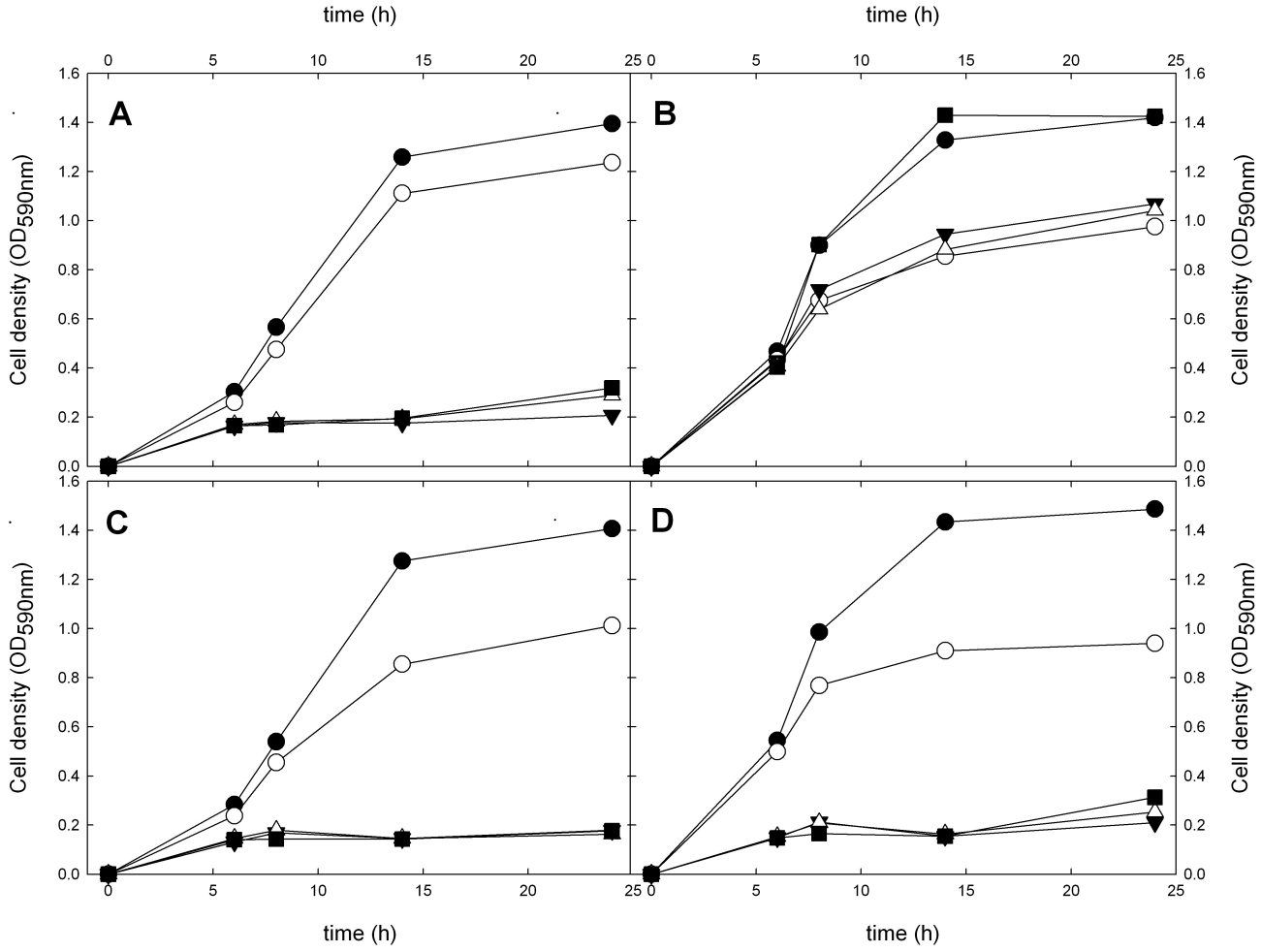


Figure 42. Growth of *Lactobacillus sanfranciscensis* TMW1.53 (panel A and B) and TMW1.53Δ*nox* (panel C and D) under static, anaerobic growth conditions (panel A and C) and under aerobic (220 rpm) conditions (panel B and D). Depicted is the time course of the increase of optical density at 590 nm (OD_{590nm}) in modified MRS medium, MRS1: ●; MRS2: ❍; MRS3: ▼; MRS4: Δ; MRS5: ■. Results are representative for three independent experiments.

L. sanfranciscensis TMW1.53 was able to grow in all five media compositions in aerated cultures, the final OD was higher in MRS1 and MRS5 supplemented with a manganese/magnesium stock solution compared to the media without Mn/Mg supplementation (panel B). *L. sanfranciscensis* TMW1.53 showed a manganese dependent growth response, the final OD and growth velocity was increased in media supplemented with manganese. *L. sanfranciscensis* TMW1.53 harbors two open reading frames coding for putative Mn^{2+}/Fe^{2+} transporters of the nramp family. The predicted gene product of *MntH1* fragment is 86% identical 94% similar to a predicted manganese Nramp protein of *P.*

pentosaceus ATCC 25745 and *MntH2* fragment is 70% identical 86% similar to a predicted manganese Nramp protein MntH2 of *L. plantarum* WCSF1. The expression of *MntH1* and *MntH2* in *L. sanfranciscensis* was verified by amplification of a 318 bp fragment of *MntH1* and a 239 bp fragment of *MntH2* from cDNA library obtained from aerobically, exponentially growing cells of *L. sanfranciscensis* TMW1.53 in MRS1. The mutant strain was only able to grow in MRS supplemented with fructose in aerated cultures during the time course; in media lacking fructose, the lag-phase was highly increased (panel D). Again as shown in panel C, the final OD was higher in MRS1 supplemented with manganese, and the growth velocity was increased compared to cultures of the mutant strain grown in MRS2. Remarkably, the growth of *L. sanfranciscensis* TMW1.53Δ*nox* under anaerobic conditions was qualitatively comparable to the growth of the mutant in aerated cultures in all five media compositions.

4.2.9 Determination of intracellular manganese levels in *L. sanfranciscensis* under static, anaerobic or aerobic conditions

The intracellular manganese content in cells of *L. sanfranciscensis* was determined for different growing conditions and media compositions at an optical density (OD_{590nm}) of 1. The results are the means ± standard deviations from three independent experiments. The manganese levels in cells of *L. sanfranciscensis* were 10.07 ± 0.8 µM ml^{-1} under aerobic conditions (220 rpm, shaking) and 12.52 ± 0.1 µM ml^{-1} under static, anaerobic conditions in MRS-1, respectively. In MRS-2 broth without supplemented manganese the contents were 9.4 ± 0.7 µM ml^{-1} under aerobic conditions, and 12.05 ± 0.1 µM ml^{-1} under static, anaerobic conditions, respectively.

4.2.10 Effect of agitation and aeration on formation of organic acids during fermentation

The growth and metabolites formed by *L. sanfranciscensis* TMW1.53 and mutant TMW1.53Δ*nox* upon growth in MRS1 were determined under anaerobic conditions and in aerated cultures (see figure 43). *L. sanfranciscensis* TMW1.53 grew to cell counts of 1.6 ± 0.55 x 10^8 cfu/ml after 12 h of fermentation, and to cell counts of 1.85 ± 0.49 x 10^8 cfu/ml after 24 h of fermentation in aerated cultures. The cognate mutant TMW1.53Δ*nox* grew to 1.2 ± 0.66 x 10^8 cfu/ml after 12 h of fermentation, and to cell counts of 1.5 ± 0.65 x 10^8 cfu/ml after 24 h of fermentation, respectively. Under static, anaerobic conditions the wild type formed a fermentation ratio of lactate to acetate of 1.79 during a fermentation time of 12 hours, after 24 hours of fermentation the ratio increased to 3.81. The formation of acetate

during static, anaerobic fermentation can be explained by the existence of oxygen in the head-space of the falcon tubes.

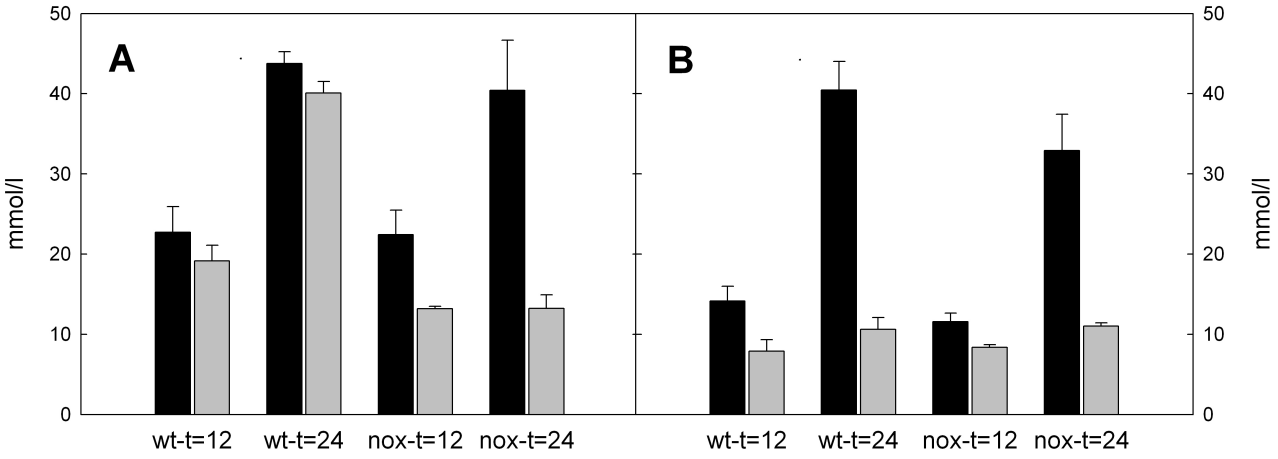


Figure 43. Formation of organic acids in aerated cultures (panel A), under anaerobic, statically conditions (panel B) after 12 hours and 24 hours of fermentation in MRS1. The concentrations of lactate (black bar) and acetate (grey shaded bar) in samples of *L. sanfranciscensis* TMW1.53 (wt) and TMW1.53Δ*nox* (nox) are given. Shown are the means ± standard deviations from five independent experiments.

The mutant TMW1.53Δ*nox* strain showed a fermentation ratio of lactate to acetate of 1.38 after a 12 hours fermentation time and a ratio of 2.98 after 24 hours of fermentation (panel B). In contrast to stationary fermented *L. sanfranciscensis* TMW1.53 cells, the wild type strain formed almost equimolar amounts of lactate and acetate under aerobic conditions. Thus, resulted in a fermentation ratio of 1.19 after 12 hours of fermentation and ended in a ratio of lactate to acetate of 1.09 at the end of the time course. The ratio of lactate to acetate in fermentations with TMW1.53Δ*nox* remained almost unaffected during the fermentation time under different oxygen proportions. Under agitation and aerobic conditions the ratio of lactate to acetate was 1.70 after 12 hours of fermentation, and resulted in a ratio of 3.05 after 24 hours of fermentation (panel A).

4.2.11 Growth of *L. sanfranciscensis* TMW1.53 and mutant TMW1.53Δ*nox* in MRS maltose medium supplemented with various fructose concentrations in aerated cultures

The growth of *L. sanfranciscensis* TMW1.53 and mutant TMW1.53Δ*nox* in MRS maltose (10 g L^{-1}) broth with various fructose concentrations under aerobic conditions (220 rpm) is shown in figure 44. Under aerobic conditions the wild type strain showed an independent growth response in regard to the availability of fructose. In contrast, growth of the mutant strain TMW1.53Δ*nox* was dependent on the availability of fructose. The mutant strain showed a

fructose dependent growth response, the growth velocity was increased in media supplemented with different amounts of fructose. Cultures of the mutant strain grown in MRS without fructose reached comparable OD-values at the end of the fermentation when 5 mM fructose was supplemented after 6 hours of fermentation.

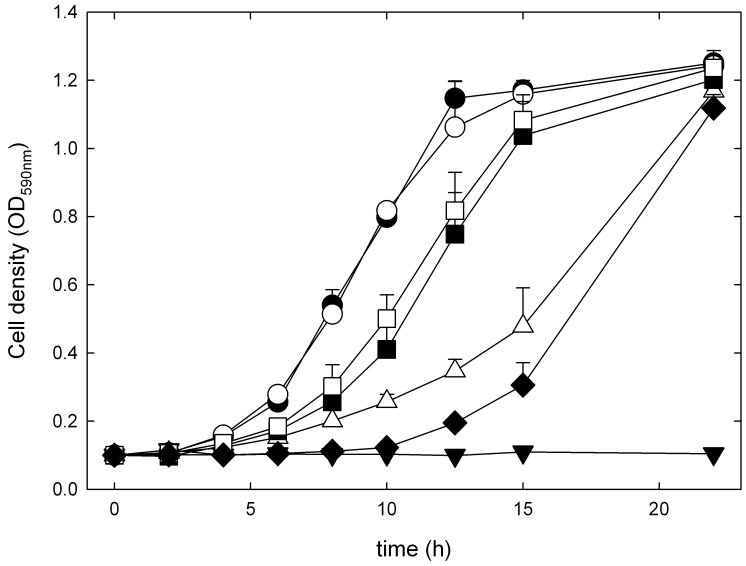


Figure 44. Growth of *L. sanfranciscensis* TMW1.53 (wt) and mutant TMW1.53Δ*nox* (nox) in MRS maltose (10 g L^{-1}) with various fructose concentrations under aerobic conditions (220 rpm). ●, wt in maltose without fructose; ❍, wt in maltose + fructose (10 mM); ▼, nox in maltose without fructose; Δ, nox in maltose + fructose (2 mM); ■, nox in maltose + fructose (5 mM); ❑, nox in maltose + fructose (10 mM); ◆, nox in maltose without fructose, after 6h + fructose (5 mM). Shown are the means ± standard deviations from three independent experiments.

The final OD-values of the mutant strain in media supplemented with fructose were comparable to the values of the wild type strain at the end of the time course. Moreover, the mutant strain TMW1.53Δ*nox* was unable to grow in media without fructose under aerobic conditions, whereas the wild type strain showed a fructose independent growth response under aerobic conditions.

4.2.12 Influence of oxidants treatment on growth inhibition of *L. sanfranciscensis* TMW1.53 and mutant TMW1.53Δ*nox* in a plate assay

To verify the protective role of Nox against oxidative stress in *L. sanfranciscensis*, the sensitivities of the wild type strain and the mutant TMW1.53Δ*nox* to oxidants, such as diamide (thiol-specific oxidant), H_2O_2 and paraquat were determined using a disc sensitivity assay. The results in table 22 indicated that the mutant strain was more sensitive to diamide treatment in comparison to the wild type strain on MRS-plates without fructose

supplementation. The influence of diamide treatment on growth inhibition of. *Lactobacillus sanfranciscensis* TMW1.53Δ*nox* (panel A) and *L. sanfranciscensis* TMW1.53 (panel B) on MRS-plates supplemented with 10 g L^{-1} maltose as the sole carbon source, could also be seen in figure 45. The diameter of inhibition for the mutant strain was determined as 35.2 ± 0.9 mm and for the wild type strain 13.1 ± 0.5 mm. The sensitivity of the mutant strain towards H_2O_2 and paraquat treatment was not so distinct in contrast to the wild type strain and the thiol specific oxidant.

Table 22. Sensitivities of *L. sanfranciscensis* TMW1.53 and mutant TMW1.53Δ*nox* to oxidants.

	Zone of inhibition (diameter, mm)									
	Wild-type					**Nox mutant**				
Oxidant	MRS1	MRS2	MRS3	MRS4	MRS5	MRS1	MRS2	MRS3	MRS4	MRS5
Diamide	23±0.9	23±1.3	22±1.4	22±1.5	25±1.3	25±2.4	21±1.2	35±1.9	32±2.2	38±2.4
Paraquat	6±0.9	6±1.3	10±1.6	6±0.6	10±1.2	10±1.7	6±1.0	16±1.5	12±1.2	18±2.0
H_2O_2	42±3.2	43±2.4	43±1.8	44±3.2	47±3.5	43±2.8	42±2.0	49±3.2	50±2.1	53±2.5

Shown are the means ± standard deviations from three independent experiments.

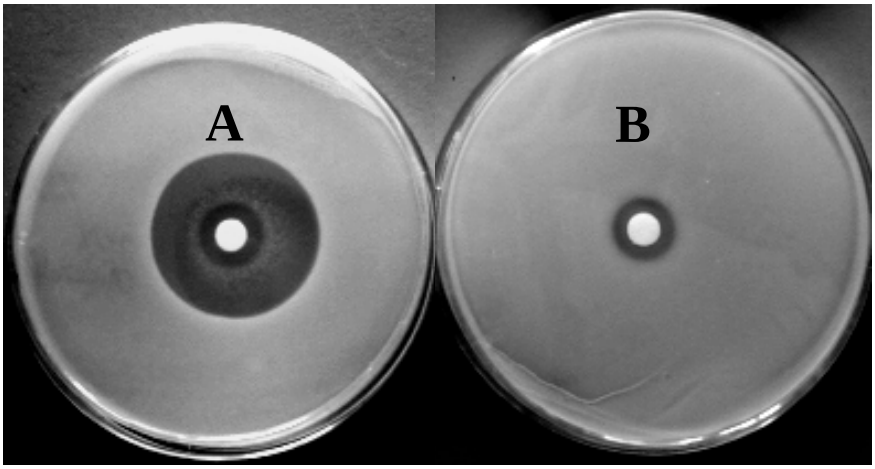


Figure 45. Influence of diamide treatment on growth inhibition of *Lactobacillus sanfranciscensis* TMW1.53Δ*nox* (panel A) and *L. sanfranciscensis* TMW1.53 (panel B) on MRS-plates supplemented with 10 g L^{-1} maltose. Shown are the zones of inhibition after two days of incubation at 30°C.

4.2.13 Contribution of the NADH oxidase (Nox) to the oxygen tolerance of *L. sanfranciscensis*

The growth response towards paraquat and diamide was verified of the wild type strain and its cognate mutant TMW1.53Δ*nox* in MRS broth. Cultures of the mutant strain grown in MRS supplemented with 90 mM paraquat without added cysteine showed a slightly more sensitive growth response in comparison to mutant strain cultures grown in MRS supplemented with cysteine. The lag-phase was increased and the growth velocity was reduced, resulted in a

decreased final OD-value (see figure 46). In contrast, growth of the wild type strain towards paraquat was unaffected in both media compositions. The mutant TMW1.53Δ*nox* showed also a distinct growth response towards diamide in MRS broth, indicated by a decreased growth rate during a five hour time course. The mutant strain showed an increased OD-value of 0.570 in MRS1 and of 0.406 in MRS2 during the time course, in comparison, the wild type strain showed an increased OD-value of 0.639 in MRS1 and 0.658 in MRS2. Thus, indicating that growth of the mutant strain was affected by diamide in culture fermentations.

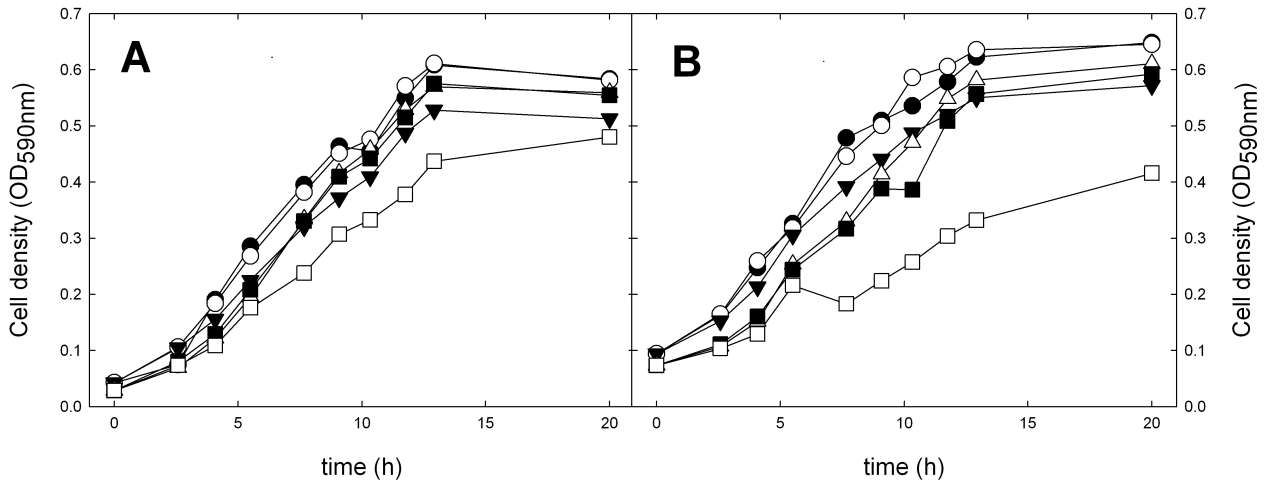


Figure 46. Growth response of *L. sanfranciscensis* TMW1.53 (wt) and mutant TMW1.53Δ*nox* (nox) in MRS supplemented with 0.5 g L^{-1} cysteine (panel A) and MRS without addition of cysteine (panel B). To the media were added different amounts of paraquat. ●, wt without paraquat; ❍, wt + 30 mM paraquat; ▼, wt + 90 mM paraquat; Δ, nox without paraquat; ■, nox + 30 mM paraquat; ❑, nox + 90 mM paraquat. Results are representative for three independent experiments.

4.2.14 Other enzymes and antioxidative compounds involved in the oxidative stress response of *L. sanfranciscensis*

Based on this work using inverse PCR and degenerated primers of known sequences for specific genes of interest involved in oxidative stress response from other bacteria, the genome sequence approach of *L. sanfranciscensis* TMW1.1304 by Vogel et al. (60), and partial genome sequence of *L. sanfranciscensis* TMW1.53 (40), respectively, a scheme is proposed for the enzymes included in the detoxification of reactive oxygen species and possible effects of antioxidative compounds in *L. sanfranciscensis*.

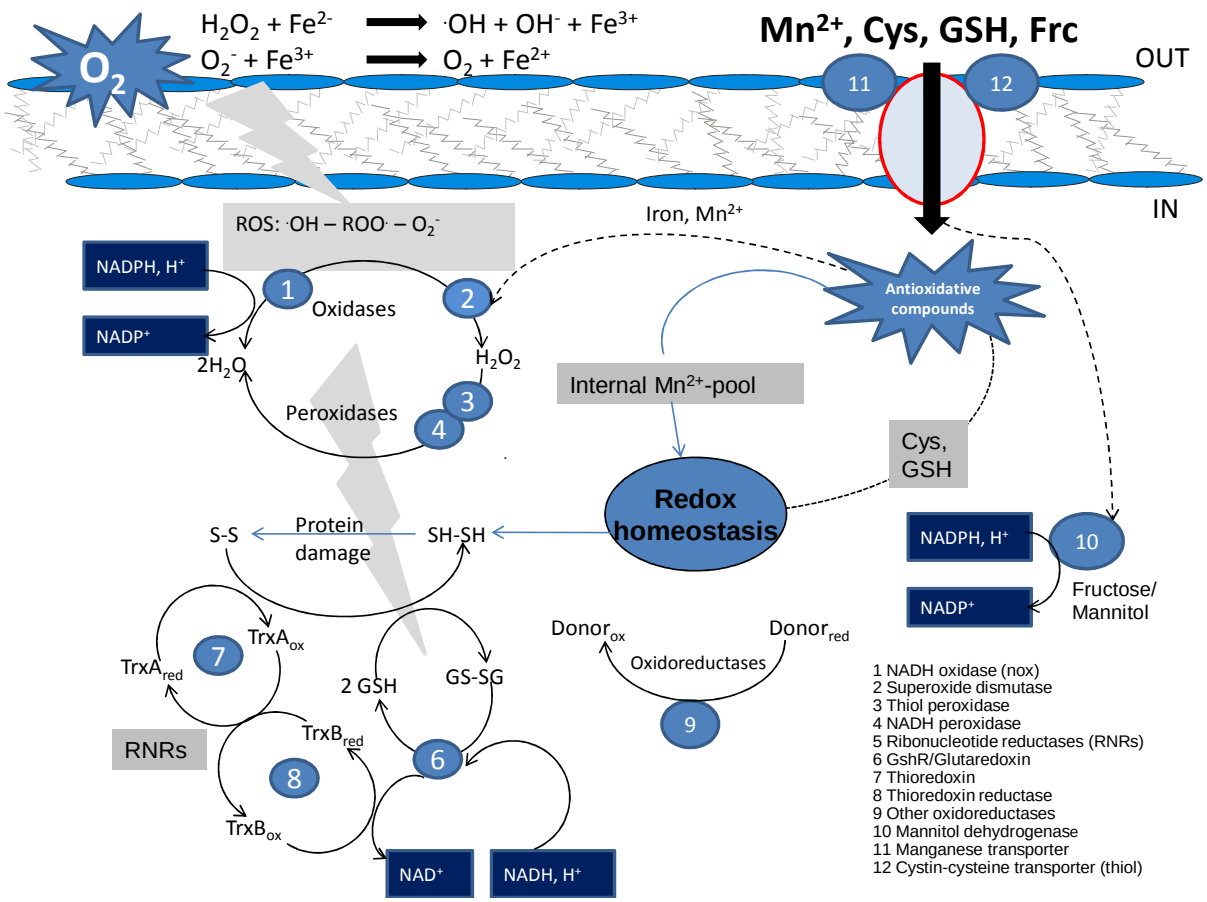


Figure 47. Insight of involved enzymes to achieve redox homeostasis in *Lactobacillus sanfranciscensis* under oxidative stress conditions (similar to (7)). Key antioxidant enzymes are indicated by numbers, a reference is included for enzymes that were characterized on genetically and/or biochemical level, the presence of other enzymes is deduced from literature data.

1. NADH oxidase (this work, from chapter 4.2.6; (22, 35, 44, 53))
2. Predicted superoxide dismutase (10)
3. Thiol peroxidase (60)
4. NADH peroxidase (this work, see chapter 5.2; (40))
5. Ribonucleotide reductases (RNRs; this work, see chapter 5.2; (40, 60))
6. Glutathione reductase/Glutaredoxin (this work, from chapter 4.2.1; (23, 40, 60))
7. Thioredoxin (this work, see chapter 5.2; (40, 60))
8. Thioredoxin reductase (this work, see chapter 5.2; (40, 60))
9. Other oxidoreductases (40, 60)
10. Mannitol dehydrogenase (this work, chapter 4.2.11; (28, 40, 60))
11. Manganese transporter (this work, from chapter 4.2.8; (60))
12. Cystin-cysteine transporter (thiol; this work, chapter 4.2.5; (60))

The proposed scheme (figure 47) showed not the all-encompassing response of *L. sanfranciscensis* due to oxidative stress, but focuses on main enzymes involved and non-enzymatic actions inside the cell, respectively, to overcome the toxic effect of reactive oxygen intermediates. *L. sanfranciscensis* do not produce typical heme-containing cytochromes and catalases (60); nonetheless, the organism is well adapted to growth in aerobic environment. The defence of aerotolerant microorganism to oxygen toxicity are enzymes transforming the intermediates, such as peroxidases, or much simpler, the involvement of oxygen in the metabolism. Several reactive oxygen species could be generated during the cellular metabolic action; oxygen is partially reduced to water leading to the formation of reactive O_2 species, e.g. superoxide anion radical (O_2^-), the hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2). These oxygen intermediates have a high oxidizing potential and thus are responsible for cellular oxygen toxicity (8, 14).

The effect of the NADH oxidase (nox) was evaluated in this study, whereas the main function of the enzyme was the regeneration of the oxidized pyridine nucleotide under aerobic conditions (see figure 42 and 43, respectively); thus, enabling the organism to produce acetate from acetyl-phosphate and the synthesis of an additional ATP (22). The genome of *L. sanfranciscensis* TMW1.1304 is harboring amongst others at least three L-lactate dehydrogenases and three D-lactate dehydrogenases (*ldh*), it was proposed that their key function is NAD^+ regeneration (60). The occurrence of a superoxide dismutase in *L. sanfranciscensis* CB1 was postulated by de Angelis et al. (10), although an analog sequence for a SodA gene is missing in the genome of *L. sanfranciscensis* TMW1.1304. All the peroxidases, e.g. thiol peroxidase, NADH peroxidase, and a putative thioredoxin reductase as well as other oxidoreductases were presented into figure 47 based on homologous sequences to other microorganism on nucleotide level (40, 60). The influence of the glutathione reductase during the aerobic life of *L. sanfranciscensis* TMW1.53 was shown in figure 40 and 41, respectively (23). The expression of the proteins, thioredoxin, thioredoxin reductase, glutaredoxin-like NrdH, and the ribonucleotide reductases NrdE and NrdF inside the cells by the wild type strain of *L. sanfranciscensis* TMW1.53 under aerobic conditions (shaking 220 rpm) was evaluated according to chapter 4.1.8. All cDNAs showed a specific PCR amplificate with primers derived from the gene sequences (see chapter 5.2, data not shown); indicating their expression under aerobic growth conditions of the strain. The transfer of antioxidative compounds, e.g. manganese, cysteine or cystine, glutathione was somehow shown in chapter 4 for *L. sanfranciscensis* TMW1.53, and on further genetically level for *L. sanfranciscensis*

TMW1.1304 (60). A proposed ability of *L. sanfranciscensis* TMW1.1304 to convert L-alanine into L-cysteine by using a cysteine desulfurase is given on genetically level (60); thus, participating in maintaining redox homeostasis during the aerobic life of the strain.

4.3 DISCUSSION

4.3.1 Influence of the glutathione reductase during the aerobic life of *L. sanfranciscensis* TMW1.53

This study considered a potential role of glutathione reductase to the oxygen tolerance of *L. sanfranciscensis*. Generally, aerobic growth of lactic acid bacteria requires the presence of catalase and / or NADH-oxidases to remove hydrogen peroxide (9, 35). Several thiol-active enzymes additionally contribute to the tolerance of lactic acid bacteria to oxygen, including the thioredoxin-thioredoxin reductase (Trx-TrxR) couple (58, 59), cyst(e)ine uptake and metabolism (56), and the glutathione-glutathione reductase system.

Streptococci harbor glutathione reductases that enable the cells to create a reducing environment and which are overexpressed during aerobic growth (41, 55, 63). Some strains of *Lactococcus lactis* accumulate glutathione in response to aerobic conditions (32). This study demonstrated that *gshR*-deficient mutants of *L. sanfranciscensis* TMW1.53 exhibited a decrease tolerance to oxygen and superoxide. Moreover, the insertional deletion of *gshR* reduced the aerobic growth rate of *L. sanfranciscensis* but did not fully eliminate oxygen tolerance in this strain. Putative thioredoxin, glutaredoxin, and thioredoxin reductase genes may serve as additional pathways to maintain intracellular redox homeostasis in the absence of an active glutathione reductase.

Oxygen tolerance in *L. sanfranciscensis* TMW1.53Δ*gshR* could be restored by addition of cysteine to the medium, indicating that the *gshR* mutant strain is more sensitive to oxidative stress because it is unable to maintain high intracellular levels of thiols. Little is known about cysteine transport systems in lactic acid bacteria; in *Saccharomyces cerevisiae*, several permeases with broad specificity contribute to cysteine transport (12). Bacterial cystine transport systems exhibit a high specificity for cystine (6, 20, 21). *L. sanfranciscensis* internalized cysteine to increase intracellular thiol levels and the rates of cysteine transport as well as the intracellular thiol levels were generally in agreement with cysteine transport in *L. reuteri* (56). Moreover, the strain expressed a gene with high homology to CyuC of *L. reuteri*. Because *L. sanfranciscensis* does not exhibit cystathionine-γ-lyase activity to liberate thiols from cystine or cysteine, it remains unclear whether cystine transport contributes to intracellular thiol homeostasis or serves nutritional requirements.

In conclusion, this study demonstrated that GshR of *L. sanfranciscensis* plays an important role to the oxygen tolerance of *L. sanfranciscensis*. It is remarkable that *gshR* homologues

were furthermore detected in several other species of lactobacilli, whereas *L. pontis*, and *L. reuteri*, species that are not capable of growth at aerobic conditions (54), are harboring no *gshR* homologue, and exhibited no GshR activity. Thus, lactobacilli differ in their use of thiol-dependent redox systems, and the contribution of the various thiol compounds to the oxygen tolerance and aerobic growth of lactobacilli may prove a relevant area of future research.

4.3.2 The influence of changes in fermentation conditions on the growth response of *Lactobacillus sanfranciscensis* TMW1.53 and mutant TMW1.53Δ*nox*

Moreover, the functional characterization of a NADH oxidase in *L. sanfranciscensis* TMW1.53 was carried out using a Nox-deficient mutant of the wild type strain. Gene regulation of *nox* could be due to catabolite regulation; the clear homology between the sequences upstream of the *L. sanfranciscensis nox* gene and the *cre* consensus sequence account for a catabolite regulation in *L. sanfranciscensis*. Catabolite regulation of *nox* makes sense in a way that the use of oxygen as electron acceptor, which is catalyzed by Nox, represents a link to the central sugar metabolism of *L. sanfranciscensis*. The question remains whether glucose or some kind of oxygen mediated regulation is the trigger for gene regulation. However, Oxy-R dependent binding sites as in the gene coding for the NADH peroxidase of *E. faecalis* (47) were not found.

Compared to the aerobic respiration of *Lactococcus lactis*, which involves the expression and regulation of several proteins in the presence of available heme (42), *L. sanfranciscensis* lacks the respiratory chain. The growth velocity and metabolite formation was not enhanced under respiratory conditions (aerobic, plus 8 µM heme, and menaquinone), done previously in our lab, thus an activated electron transport chain could be excluded in *L. sanfranciscensis*. The non-existence of genes essential for respiratory metabolism could be supposed in *Lactobacillus sanfranciscensis*, e.g. cytochrome oxidase, menaquinone biosynthesis and heme uptake.

The flavoprotein NADH oxidase of *L. sanfranciscensis* catalyses the direct reduction of O_2 to H_2O by NADH (44). In turn, aerobic growth of *L. sanfranciscensis* in different MRS-media compositions was found to result in a higher final cell yield and growth rate than anaerobic growth, suggesting more efficient production of ATP during aerobic growth, as also described by Stolz et al. 1995 (53). *L. sanfranciscensis* has been shown to use the acetate branch whenever electron-acceptors such as fructose or oxygen are available, thereby increasing its energy and growth yield. The wild type strain could grow in all five media-compositions with an enhanced growth velocity and final OD-value in media supplemented with manganese

under aerobic conditions. In aerated cultures, the availability of fructose was not a limiting growth factor, in contrast to higher cell yields in cultures incubated with manganese. Thus, indicating the role of Mn^{2+} in the detoxification of superoxide by superoxide dismutase (SodA) (10), and also the non-enzymatic detoxification of free radicals (25). For example, *L. plantarum* maintains a remarkably high cytoplasmic concentration of Mn^{2+}, but is essentially devoid of iron and heme (2), it is suggested that a large portion of Mn^{2+} is 'free' in a chemical relevance and substitutes functionally for iron and heme (25). Thus, the lesser amount of intracellular manganese in aerobically grown cells of *L. sanfranciscensis* could count for the fact, that 'free' manganese provides a basis for a chemical quenching agent of radicals. An enhanced manganese-coupled SodA activity could not be seen in *L. sanfranciscensis* CB1, when the cells were cultivated in the presence of 0.1 mM paraquat (10). The two manganese transporters MntH1 and MntH2 could be essential for Mn^{2+} homeostasis in *L. sanfranciscensis* TMW1.53.

The mutant strain TMW1.53Δ*nox* showed a fructose-dependent growth response under aerobic conditions, by lacking an active Nox enzyme the strain was unable to use oxygen as an electron acceptor. In contrast to the wild type strain, the mutant grew only in MRS supplemented with fructose, by using the carbohydrate as an external electron acceptor. The availability of an external electron acceptor will decide whether ethanol or acetate (+ATP) is formed. Lucey and Condon assumed that the ethanol branch is no more than a salvage route, permitting growth when an external electron acceptor is not available (37).

The shut-down of the ethanol branch of the phosphoketolase pathway in the presence of oxygen seems to be very common among heterofermentative lactic acid bacteria. Acetyl phosphate is a key metabolic intermediate in the heterofermentative pathway of *L. sanfranciscensis*, representing a branching point of the carbon flux (27). The increased acetate/lactate ratio accumulated during fermentation of *L. sanfranciscensis* under aerobic conditions showed that an electron balance was achieved by using oxygen as an external electron acceptor. The mutant strain was unable to regenerate reduced coenzymes in form of NADH by Nox in aerated cultures, and resulted in a decreased acetate/lactate ratio. The carbon flux was directed towards the reduced end product ethanol when reduced coenzymes were regenerated. Some heterofermentative lactic acid bacteria ferment fructose, using it both as a growth substrate and as an electron acceptor (18, 33, 48), *L. sanfranciscensis* used fructose exclusively as an external electron acceptor. The mutant strain TMW1.53Δ*nox* showed that with an increase of fructose concentration in the medium, the same maximum

OD value was achieved in comparison to the wild type strain at the end of the fermentation. However, growth was fully impaired in medium without added fructose. This study additionally considered a potential role of NADH oxidase from *L. sanfranciscensis* to overcome oxygen toxicity. Generally, aerobic growth of lactic acid bacteria requires the presence of catalase, superoxid dismutase, and / or NADH-(per)oxidases to remove reactive oxygen species (9, 36). Several thiol-active enzymes additionally contribute to the tolerance of lactic acid bacteria to oxygen, including the thioredoxin-thioredoxin reductase (Trx-TrxR) couple (58, 59), cyst(e)ine uptake and metabolism (56), and the glutathione-glutathione reductase system (23). This study showed that the mutant strain TMW1.53Δ*nox* exhibited a decrease tolerance to thiol specific oxidative stress and superoxide. The inhibition of growth upon diamide treatment on MRS-plates could be restored by supplementation of fructose; in cultures of the mutant strain, the inhibition of diamide could be restored by manganese supplementation. Recently it was shown, that *Candida albicans* induced several oxidoreductases after treatment with the thiol oxidizing agent (30). Moreover, the insertional deletion of *nox* reduced slightly the growth rates after treatment with paraquat in MRS without added cysteine. The growth deficit could be restored by addition of cysteine. *L. sanfranciscensis* internalized cysteine to increase intracellular thiol levels; the rate of cysteine transport, as well as the intracellular thiol levels were generally in agreement with cysteine transport in *L. reuteri* (56). Moreover, the strain expressed a gene with high homology to CyuC of *L. reuteri* (23).

In conclusion, for heterofermentative lactic acid bacteria, it has been observed that under aerobic conditions the growth yield, as well as the ATP yield, was markedly higher than that under anaerobic conditions. The balance between attempting to attain maximal energy yields and maintaining redox balance is shifted in favor to the energy gain, when *L. sanfranciscensis* transfers the electrons to external electron acceptors such as fructose or oxygen. The importance of NADH oxidase is likely to lie in maintaining the intracellular redox balance, thus assuring fast aerobic growth by the consumption of NADH. The essential function of Nox is that it allows O_2 to act as a terminal electron acceptor, just like in more conventional, but much more complex respiratory systems (42). Interestingly, the fact that *L. sanfranciscensis* exhibits one of the smallest genomes approximately (1.3 Mb) (11, 60) is accompanied by a one enzyme minimalistic strategy to improve energy yield under aerobiosis. Several other species of lactobacilli like *L. pontis*, *L. reuteri*, *L. fermentum* and *L. amylovorus* are described not being able to grow under aerobic conditions (54). The defense

of *L. sanfranciscensis* to oxygen toxicity is the involvement of oxygen in the metabolism by Nox-activity. Thus, lactobacilli differ in their response to oxygen, and their ability to use O_2 in central carbon flux may prove a relevant area of future research.

4.3.3 Insight into the oxidative stress response of *L. sanfranciscensis*

The microorganism is lacking heme and is devoid of cytochromes, exceptional is a gene for cytochrome d ubiquinol oxidase subunit 1 in the genome of *L. sanfranciscensis* TMW1.1304 (60). Moreover, the observation of even weak catalase activity is not notable (data not shown), which has been shown to be associated with a class of catalases that is independent of a heme requirement (62). The activity of non-heme catalase was found to be widespread among strains of lactobacilli (13); a non-heme catalase of *L. plantarum* ATCC14431 was heterologously expressed in several lactobacilli, but was found to be only active in *L. casei*; thus, the result was attributed to the comparable internal manganese levels inside both strains, whereas the other genetically modified LAB exhibited a 10- and 100-fold lesser internal amount of manganese (45). The co-expression of superoxide dismutase and catalase provides extensive oxidative stress resistance in *L. rhamnosus*; upon addition of 10 mM H_2O_2, the survival ratio of the recombinant strain was 400-fold higher than that of the wild type strain (1). Furthermore, the expression of a recombinant manganese SodA gene in lactobacilli provides protection against hydrogen peroxide toxicity (5); the activity was pronounced in *L. reuteri* compared to the other strains due to the availability of sufficient manganese ions inside the cell. The maintenance of high intracellular Mn-levels in *L. reuteri* could be guaranteed by Mn(II)/Fe(II) transporter (Accession number: AE014283), and a putative proton-dependent manganese transporter (Accession number: AY267207). Both transporters belong to the MntH or Nramp superfamily, similar to the presented manganese transporters for *L. sanfranciscensis* in chapter 4.2.8. An active superoxide dismutase for *L. sanfranciscensis* CB1 was postulated by de Angelis et al. (10), although in the genome approaches no homologous sequence of SodA was observed in *L. sanfranciscensis* TMW1.53 (40) and *L. sanfranciscensis* TMW1.1304 (60), respectively. A study by Archibald and Fridovich (1981) demonstrated that lactic acid bacteria, which contained high intracellular levels of Mn^{2+}, were devoid of true superoxide dismutase activity. Conversely, those which possessed true SodA activity did not contain high levels of Mn^{2+}. It was proposed that high Mn^{2+}-levels represents a substitution for SodA activity (3). The determination of intracellular manganese levels in *L. sanfranciscensis* TMW1.53 was shown in chapter 4.2.9, and already discussed in chapter 4.3.2; thus, the adaption of high intracellular levels of manganese

resultant from life in manganese rich media (flour, plant sources) could lead to an increased oxygen tolerance in *L. sanfranciscensis*.

As mentioned in chapter 1, NADH peroxidases or thiol peroxidases resume the role of heme-containing catalases by reducing intracellular levels of H_2O_2, while also regenerating oxidized pyridine nucleotide (20, 47). The sequence of a glutathione peroxidase was derived from alignments with known sequences from the database for *L. sanfranciscensis* TMW1.1304 (60), and a sequence for NADH peroxidase was generated by using inverse PCR technique (see chapter 5.2 and (40)) in *L. sanfranciscensis* TMW1.53. Both possible enzymes could have a main impact in detoxification of H_2O_2 in *L. sanfranciscensis* as shown in figure 47; the peroxidases might have exceed importance in maintaining redox homeostasis compared to GshR (23) due to the absence of a heme-catalase in the genome of *L. sanfranciscensis* (60).

The ability to respond to reactive oxygen intermediates requires mechanism to minimize the occurrence of thiol oxidation (figure 47); the influence of the glutathione reductase to the aerobic life of *L. sanfranciscensis* TMW1.53 was presented earlier in this thesis (see also (23)); the genome of *L. sanfranciscensis* TMW1.1304 contain no homologous genes for γ-glutamylcysteine synthetase (*gshA*) and glutathione synthetase (*gshB*), indicating that glutathione has to be imported from the medium (60). A complete glutathione system for *L. fermentum* was presented by Kullisaar et al. (29); glutathione synthesis, uptake and redox turnover ability arranged in *L. fermentum* lead to an increase in tolerance against oxidative stress of the strain. Glutathione was found to provide protection against exposure to H_2O_2 and is most likely functional as a scavenger of reactive oxygen intermediates (51). Other studies revealed that the protective role of reduced glutathione against cryodamage of the cell membrane of *L. sanfranciscensis* is partly due to preventing peroxidation of membrane fatty acids and protecting Na(+),K(+)-ATPase (64). Moreover, it is proposed that glutathione could protect certain key metabolic enzymes via S-thiolation against cold stress in *L. sanfranciscensis*; protein SH groups form mixed disulfides with low-molecular weight thiols. Another predicted protective mechanism of glutathione against cold stress is its role in preventing intracellular pH-drop; *L. sanfranciscensis* cells cultivated in broth supplemented with glutathione showed a significant increase in intracellular pH during the cold-treated process for 30 days in comparison to cells without added glutathione (65). The inactivation of a gene responsible for glutathione production in *L. plantarum* WCFS1 affected survival of the strain under H_2O_2 stress; an overlap between the glutaredoxin and thioredoxin system was postulated for *L. plantarum* using DNA-microarrays (51). Extracellular low-molecular weight

thiols, e.g. glutathione, cystine, and cysteine, respectively, were demonstrated to be the major contributors to changes in the redox potential (Eh). The possible role of changes in thiol concentrations inside and outside of cells in the processes of signal transduction and redox regulation of cellular functions was discussed by Oktyabrskii et al. (39). It was proposed that in tested bacterial cultures like *E. coli*, glutathione and cysteine may be responsible for the major contribution to the generation of Eh leaps.

Furthermore, on the basis of sequence similarity, a glutaredoxin-like protein, two thioredoxin reductases, a putative thioredoxin peroxidase, and three thioredoxin-like proteins were identified in the genome of *L. sanfranciscensis* TMW1.1304 (60). Some of these genes were also found in *L. sanfranciscensis* TMW1.53 (see chapter 4.2.14 and 5.2); their impact remains to be evaluated during aerobic conditions, but the expression was already shown in broth fermentations. The overexpression of gene trxB1 resulted in a 3-fold overproduction of thioredoxin reductase (TR) activity and higher resistance toward diamide and H_2O_2 in *L. plantarum* WCFS1. Disruption of trxB1 resulted in a cognate mutant strain with decreased TR activity (2.5-fold) and in a 19% lower growth rate under aerobic conditions compared to the wild-type (51). Thus, both systems, glutathione-glutathione reductase and thioredoxin-thioredoxin reductase, respectively, are playing a crucial role in resistance towards oxidative stress in lactobacilli (see also figure 47). Redox-sensitive amino acid side chains such as cysteine-thiols that monitor redox signals including oxygen, cytoplasmic redox state or the production of reactive oxygen species are common in bacteria beside other specific sensors (16). Redox sensors control the processes that function to maintain redox homeostasis, usually at the level of transcription. The OxyR transcription factor is a key regulator of the *E. coli* response to oxidative stress (61). The genome approach of *L. sanfranciscensis* TMW1.1304 revealed a redox-sensing transcriptional repressor (*rex*) (60), his function during aerobic conditions or in regeneration of pyridine nucleotide seems to be another trigger for *L. sanfranciscensis* to cope environmental stress.

In addition, genes for ribonucleotide reductases *nrdI*, *nrdH* (glutaredoxin-like), *nrdE*, and *nrdF* were observed in the genome of *L. sanfranciscensis* TMW1.53 (this work and (40); the operon consists of a gene cluster *nrdHEF* (see chapter 5.2). As mentioned in chapter 4.2.14, those genes were expressed during aerobic growth of the strain in fermentation broth. Staphylococci contain a class Ib NrdEF ribonucleotide reductase (RNR) that is responsible for the synthesis of deoxyribonucleotide precursors for DNA synthesis and repair under aerobic conditions. The genes encoding RNR are contained in an operon consisting of three genes,

nrdIEF, whereas many other class Ib RNR operons contain a fourth gene, *nrdH*, that determines a thiol redoxin protein named NrdH (43). *In vitro*, *S. aureus* NrdH was found to be an efficient reductant of disulfide bonds in low-molecular weight substrates. Moreover, its ability to reduce NrdEF is comparable to that of thioredoxin-thioredoxin reductase (43). The ribonucleotide reductase system of *L. lactis* was presented by Jordan et al. (24), they showed that the system consist of an NrdEF-type enzyme, and a small electron transport protein NrdH. The NrdEF enzymes use NrdH proteins as electron transporter in place of thioredoxin or glutaredoxin used by NrdAB enzymes. The impact of RNR during the aerobic life of lactobacilli involved in glutaredoxin-like and thioredoxin-thioredoxin reductase systems may prove also a relevant area of future research due to non-existence of scientific work related to this phenomenon.

In conclusion, as proposed in figure 47, *L. sanfranciscensis* is well adapted to maintain redox-homeostasis during oxidative stress. The influences of some key enzymes involved in detoxification of oxygen intermediates were evaluated based on cognate knockout mutants of *L. sanfranciscensis*. An almost complete glutathione system is existent in *L. sanfranciscensis*; composed of glutathione reductase, peroxidase, and glutaredoxin-like proteins, only genes for the synthesis of GSH are missing in the genome of *L. sanfranciscensis*. Uptake of thiol substrates from the environment are accomplished with the help of several transporter systems. Nonetheless, several more factors have to be elucidated, e.g. peroxidases, thioredoxin reductase-thioredoxin system, RNR, and redox sensors inside the cells of *L. sanfranciscensis* to get an overall picture of the oxidative stress response.

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CONCLUDING REMARKS

The comprehensive aim of this thesis was to find and characterize metabolic activities of lactobacilli that are relevant in wheat sourdough fermentations and contribute to the final bread aroma and volume, respectively. This aspect was outlined in the title of this thesis "Contribution of thiol- and hydroxycinnamic acids metabolism of sourdough lactobacilli on structural and sensorial properties of wheat breads". Moreover, an insight was given into the oxidative stress response of *L. sanfranciscensis*; this germ is the predominant microorganism in type-I-sourdough fermentations. In the early phase of evolvement, sourdough contains a lot of dissolved oxygen; it could be also introduced into the dough during the kneading. In both situations, an oxygen tolerant microorganism has advantages in competition as compared to strictly anaerobic organisms. It should be mentioned that in all chapters of this thesis focus was laid on *L. sanfranciscensis*. Nonetheless, screening approaches in chapter 3 confirmed that *L. sanfranciscensis* is not capable to generate volatile aroma odors in fermentations with phenolic compounds. Therefore, other lactobacilli were utilized to find enzyme activities able to generate volatile aroma compounds from hydroxycinnamic acids.

The importance of gluten proteins for the baking quality of wheat flours is explained by their ability to form high molecular weight aggregates that are stabilised by covalent bonds and non-covalent interactions between monomers. The use of sourdough in wheat baking improves the sensorial and nutritional quality of the products. Especially proteolytic events during fermentation are highly relevant for the aroma formation in wheat breads; however, the amount of the glutenin macropolymer is strongly reduced during sourdough fermentations. Lactobacilli from sourdough were characterized with respect to their glutathione reductase (GshR) activity; *L. sanfranciscensis*, the cognate mutant Δ*gshR*, and two strains (*L. reuteri* and *L. pontis*) that are GshR–negative, respectively, were used to evaluate their impact on thiol levels during wheat sourdough fermentations. *L. sanfranciscensis* increased thiol levels in wheat doughs, whereas a decrease of thiols was observed in wheat doughs fermented with *L. sanfranciscensis* Δ*gshR*. The extracellular accumulation of thiols is particularly relevant in wheat doughs. The quality and quantity of gluten proteins in wheat flours is of paramount importance for wheat bread quality, and the intermolecular disulfide crosslinks of glutenin subunits to form the glutenin macropolymer are dependent on the presence or absence of low molecular weight sulfhydryl compounds. The direct impact of the glutathione reductase of *L. sanfranciscensis* on gluten proteins during sourdough fermentations was shown in figure 21.

The degradation of the glutenin fraction was deviating by approx. 20% between the wild type strain of *L. sanfranciscensis* and the mutant TMW1.53Δ*gshR*. Thus, the knockout of a single enzyme activity resulted in a decreased glutenin network breakdown during wheat sourdough fermentations. It should be noted that attention should focus not only on single enzyme reactions by the sourdough microbiota, but also on an overall screening approach of different lactobacilli in regard to redox potential during wheat sourdough fermentations. Thus, an overall perception concerning the reductive or oxidative potential of lactobacilli during fermentations could lead to the best selection of fermentation microorganism in respect to the desired quality parameter.

Crucial results were obtained in respect to the content of ferulic acid during wheat sourdough fermentations (see figure 24) while performing the analyses for the above mentioned determinations on thiol levels; thus, resulted in the examinations of chapter 3 “Distribution of enzyme activities in lactobacilli able to generate volatile aroma compounds from hydroxy-cinnamic acids”. The liberation of ferulic acid during wheat sourdough fermentations by *L. sanfranciscensis* was the starting point, and led to the following questions: Are lactobacilli able to release bonded ferulic acid in arabinoxylan during wheat sourdough fermentations? Is this a strain specific phenomenon? Is the liberation of ferulic acid during fermentations achieved by microbial or exogenous enzyme activities? Is the redox potential in the dough system the main factor for ferulic acid release during sourdough fermentations? What are the fates of free forms of phenolic acids in fermentations? By using database analysis the sequenced *Lactobacillus* genomes were examined for putative arabinoxylan degrading enzymes to identify distinctive features for the metabolism of plant cell wall backbone in lactobacilli. The cascade for generating volatile aroma compounds by degradation of arabinoxylan should be composed of the following enzymes: 1. endo-1,4-β-xylosidase and xylanase, 2. α-L-arabinofuranosidase, 3. cinnamoyl esterase, 4. phenolic acid decarboxylase (see also figure 23). *L. brevis* TMW1.1326 encoded several proteins with the ability to hydrolyze glycosylated plant secondary metabolites. The characterization of the recombinant α-L-arabinofuranosidase and β-xylosidase, respectively, confirmed active enzymes with the ability to degrade water-soluble wheat arabinoxylan. Cinnamoyl-esterase activity of lactobacilli was determined using a plate screening approach with ethylferulate as the substrate; the free form of hydroxycinnamic acids is the requirement for gaining volatile aroma compounds during fermentations. The physiological tests for predicted cinnamoyl esterase activity could be ensured on a genetically background. Predicted esterases of

lactobacilli able to hydrolyze esterifies hydroxycinnamates were heterologously expressed and characterized. Moreover, the fate of the free liberated ferulic acid was verified by a PCR-screening for phenolic acid decarboxylase genes in selected lactobacilli. In fermentation studies with lactobacilli using mMRS broth supplemented with ferulic acid and coumaric acid, respectively, the fermentation supernatants were analysed by HPLC to determine the conversion of the substrates to volatile aroma compounds, e.g. 4-vinyl-2-methoxyphenol. Moreover, a combination of *L. brevis* TMW1.1326 and *L. pontis* TMW1.1086 showed capable results related to the generation of active aroma odors. With the culture combination and wort as the fermentation substrate, almost 11 µM 4-vinylguaiacol and 60 µM 4-vinylphenol were accumulated after 120 hours. Thus, the selection of strains capable of generating aroma active substances in wheat sourdough fermentations and the best choice of wheat cultivar in respect to phenolic contents could lead to aroma enhancement. A combination of strains for substrate fermentations seems to have potential in regard to the ability to liberate improved aroma doughs.

Finally, an insight of the oxidative stress response in *Lactobacillus sanfranciscensis* was given; two main enzyme activities NADH oxidase and glutathion reductase, respectively, were examined in *L. sanfranciscensis* and their role in maintaining redox homeostasis was elucidated. This study demonstrated that GshR of *L. sanfranciscensis* plays an important role to the oxygen tolerance of *L. sanfranciscensis* (see chapter 4). An almost complete glutathione system is existent in *L. sanfranciscensis*; composed of glutathione reductase, peroxidase, and glutaredoxin-like proteins, only genes for the synthesis of GSH are missing in the genome of *L. sanfranciscensis*. Uptake of thiol substrates from the environment are accomplished with the help of several transporter systems. Moreover, for heterofermentative lactic acid bacteria, it has been observed that under aerobic conditions the growth yield, as well as the ATP yield, was markedly higher than that under anaerobic conditions. The balance between attempting to attain maximal energy yields and maintaining redox balance is shifted in favor to the energy gain, when *L. sanfranciscensis* transfers the electrons to external electron acceptors such as fructose or oxygen. The importance of NADH oxidase is likely to lie also in maintaining the intracellular redox balance, thus assuring fast aerobic growth by the consumption of NADH. The essential function of Nox is that it allows O_2 to act as a terminal electron acceptor, just like in more conventional, but much more complex respiratory systems. *L. sanfranciscensis* is lacking heme and is devoid of cytochromes, exceptional is a gene for cytochrome d ubiquinol oxidase subunit 1 in the genome of *L. sanfranciscensis* TMW1.1304. A higher growth yield

for *L. sanfranciscensis* under aerobic conditions in medium supplemented with heme and menaquinone, respectively, could not be seen in comparison to normal nutrient broth (data not shown). Thus, an activation of a respiratory chain in *L. sanfranciscensis* was not observed.

5

5. APPENDIX

5.1 GENERAL MATERIALS AND METHODS

5.1.1 Used equipment in the studies according to chapter 2, 3, and 4.

Table 23. Equipment

Equipment	Model	Supplier
Anaerocult devices	Anaerobic jar	Merck Millipore
Analytical balance	CPA	Sartorius, Scaltec Instruments
Autoclave	2540 ELV, Varioklav	Systec GmbH, H+P Labortechnik
Blotting oven	MINI 10	MWG Biotech AG
Centrifuge	Sigma 1K15, 6-16K	Sigma Labortechnik
	J-6, J-2	Beckman
	Hermle Z383K, Z382K	Hermle Labortechnik
Electroporation system	Bio-Rad Gene pulser	Bio-Rad Laboratories
Fluorescence microscopy	Stereomikroskop	Carl Zeiss GmbH
FPLC	Biologic HR	Bio-Rad Laboratories
	Model 2128 Collector	Bio-Rad Laboratories
Gel chamber	Easy Cast	Owl Separation Systems
Gel documentation	Herolab	Herolab
HPLC	Ultimate 3000	Dionex
Incubator	Certomat	B. Braun Biotech
	Heraeus B5042E	Heraeus Instruments
	Memmert INB	Memmert GmbH & Co. KG
Laminar flow	HERA safe	Heraeus Instruments
Luminescence Spectrometer	LS 50B	Perkin Elmer
Microscope	Axiolab	Carl Zeiss GmbH
MQ water	Euro 25, RS90-4/UF pure water system	SG Wasseraufbereitung GmbH
Nanodrop	Nanodrop 1000	Peqlab Biotechnologie GmbH
PCR-Cycler	Primus 96 Plus	MWG Biotech AG
	Mastercycler gradient	Eppendorf AG
	Cycler	Applied Biosystems
pH electrode	InLab 412	Mettler-Toledo
pH device	Knick pH 761	Knick
Photometer	Novaspellq	Pharmacia Biotech
Pipettes	Pipetman	Gilson-Abomed
Plate readers	TECAN Spectrafluor	TECAN GmbH
	TECAN Sunrise	TECAN GmbH
Power supplies	MPP 2x3000 Power S.	MWG Biotech AG
	Electrophoresis PS EPS3000	Pharmacia Biotech

Rotary evaporator	VV2000	Heidolph
Sequencer	Model 377A	Applied Biosystems
SDS-PAGE	Mini Protean III-System	Bio-Rad Laboratories
Shaking devices	Certomat R	B. Braun Biotech International
	Vortex 2 Genie	Scientific Industries Inc.
Spiral plater	-	IUL
Stirrer	-	IKA Labortechnik
Thermo block	Techne DRI-Block DB3	Thermo-Dux GmbH
Ultrasonic bath	Sonorex Super RK 103H	Bandelin electronic
Ultra sonification	UP 200S	Dr. Hielscher GmbH
	SONOPLUS/SH70G	Bandelin electronic
UV table	Herolab UVT 28M	Herlab GmbH Laborgeräte
Vacuum controler	-	KNF Neuberger
Water bath	Lauda BD	LAUDA Dr. Wobser GmbH

5.1.2 Expendable materials

Other materials used in the studies according to chapter 2, 3, and 4.

Table 24. Other materials

Material	**Type**	**Supplier**
Anaerocult	C mini, A	Merck
Electroporation cuvettes	-	Biozym Scientific GmbH
Filter, sterile	Filtropur S 0.2 µm	Sarstedt
HPLC column	C18 rp 100 x 4.6 mm	Phenomenex Ltd.
	Polyspher OAKC column	Merck
Microtiter plates	Multi well 96 flat bottom	Sarstedt
Nylon membranes	-	Amersham Biosciences
PCR reaction tubes	100 µl	Sarstedt
Petry dishes	92 x 16 mm	Sarstedt
Pipet tips	2 µl, 10 µl, 200 µl, 1000 µl	Peqlab
Reaction tubes	2 ml, 1.5 ml, 200 µl	Eppendorf
Sensi-discs	sterile	Becton Dickinson GmbH
Tubes, sterile	5 ml, 15 ml, 50 ml	Sarstedt
TLC Silica gel	TLC 60 WF $_{254S}$	Merck
VISKING dialysis tubing	20/32	SERVA
FPLC columns	HisTrap HP	Amersham Biosciences

5.1.3 Kits

Kits used in the studies according to chapter 2, 3, and 4.

Table 25. Commercially available kits

Kit	**Type**	**Supplier**
DIG-DNA Labeling Mixture	Southern hybridization	Roche Diagnostics
E.Z.N.A. Bacterial DNA Kit	DNA isolation	Omega Bio-Tek Inc.
GenomeLab DTCS-Quick Start Kit	Sequencing	Beckman Coulter
KOD hot start DNA polymerase	DNA polymerase	Novagen EMD chemicals
PeqGold plasmid miniprep kit	Plasmid miniprep	PEQLAB Biotechnologie
PeqGold gel extraction kit	Gel extraction	PEQLAB Biotechnologie
QIAquick PCR purification kit	PCR purification	Qiagen GmbH

RNeasy Mini kit	RNA isolation	Qiagen GmbH
Wizard Plus SV Minipreps DNA	Plasmid isolation	Promega

5.1.4 Chemicals

Chemicals used in the studies according to chapter 2, 3, and 4.

Table 26. Chemicals

Chemical	**Purity**	**Supplier**
6 x DNA loading dye	-	Fermentas GmbH
Acetic acid	99-100%	Merck
Acetone	HPLC grade	Carl Roth GmbH
Acetonitril	HPLC grade	Carl Roth GmbH
Acrylamid-bis solution	30 % (w/v)	SERVA
Agar	-	Difco
Agarose	-	Biozym Scientific GmbH
Ampicillin sodium salt	93.3%	Gerbu Biotechnik GmbH
Ammonium chloride	≥ 99.5% p.a.	Carl Roth GmbH
Ammonium persulfate	Electrophoresis grade	SERVA
Arabinan	~ 95%	Megazyme
Arabinose	> 98%	Sigma-Aldrich
Arrow Taq-polymerase	-	MP Biomedical
Arrow Taq-polymerase	-	Invitrogen
Bio-Rad Protein assay	-	Bio-Rad Lab. GmbH
Biotin	~ 98%	Sigma-Aldrich
Boric acid	≥ 99.5%	Carl Roth GmbH
Bromphenol blue	Electrophoresis	Sigma-Aldrich
BSA	fraction V for bio use	Merck
$CaCl_2$ x 2 H_2O	p.a.	Merck
Caffeic acid	-	Sigma-Aldrich
Calcein	-	Sigma-Aldrich
Chloroform	p.a.	Merck
Citric acid	p.a.	Sigma-Aldrich
Cobalamine	p.a.	Sigma-Aldrich
Coumaric acid	-	Sigma-Aldrich
Cyanocobalamine	p.a.	Sigma-Aldrich
Cycloheximide	-	Sigma-Aldrich
Cystathionine	-	Sigma-Aldrich
Cysteine hydrochloride	p.a.	Merck
Diamide	-	Sigma-Aldrich
Di-ammonium hydrogen citrate	p.a.	Merck
Dithiothreitol	-	Sigma-Aldrich
DMSO	≥ 99.5% p.a.	Carl Roth GmbH
DNA Polymerase	-	Promega
dNTP	-	MP Biomedical, Invitrogen
DTNB	-	Sigma-Aldrich
DTT	high purity	Gerbu Biotechnik GmbH
EDTA	for molecular biology	Sigma-Aldrich
Erythromycin	-	Sigma-Aldrich
Ethanol, denatured	99% with 1% MEK	Laborbedarf Nierle

Ethanol, absolute	≥ 99.8%	VWR
Ethidium bromide	1% in H_2O	Merck
Ethyl acetate	p.a.	Merck
Ethyl ferulate	98%	Sigma-Aldrich
FD restriction buffer	-	Fermentas GmbH
FD restriction enzymes	-	Fermentas GmbH
Ferulic acid	99%	Sigma-Aldrich
Folic acid	p.a.	Sigma-Aldrich
Formic acid	p.a.	Sigma-Aldrich
Fructose	for biochemical use	Sigma-Aldrich
Glucose	for biochemical use	Sigma-Aldrich
Glycerol	99.5% high purity	Gerbu Biotechnik GmbH
Glycine	p.a.	Merck
GSH	-	Sigma-Aldrich
GSSG	-	Sigma-Aldrich
H_2O_2	-	Sigma-Aldrich
HCl 37%	p.a.	Merck
HEPES buffer	-	Sigma-Aldrich
Imidazole	for biochemical use	Sigma-Aldrich
IPTG	p.a.	Gerbu Biotechnik GmbH
Iron sulfate heptahydrate	p.a.	Fluka Chemie GmbH
Isopropanol	p.a.	Scharlau Chemie S.A.
KH_2PO_4	p.a.	Carl Roth GmbH
K_2HPO_4 x $3H_2O$	p.a.	Merck
Kalium acetate	p.a.	Merck
KCl	p.a.	Merck
Ketobutyrate	-	Sigma-Aldrich
KPi buffer	-	Sigma-Aldrich
Lactic acid	p.a.	Sigma-Aldrich
Lysozyme	-	SERVA
2-Mercaptoethanol	-	Sigma-Aldrich
Maltose	for biochemical use	Merck
Meat extract	-	Merck
MES buffer	-	Sigma-Aldrich
Methanol	HPLC-grade	Mallinckrodt Baker B.V.
Methyl caffeate	-	Sigma-Aldrich
Methyl coumarate	-	Sigma-Aldrich
Methyl-tert-butylether	-	Oxeno GmbH
$MgCl_2$ x 6 H_2O	p.a.	Merck
$MgSO_4$ x 7 H_2O	p.a.	Merck
$MnCl_2$	p.a.	Merck
$MnSO_4$ x 4 H_2O	p.a.	Merck
MTT	-	Sigma-Aldrich
NaCl	p.a.	Merck
NADH	-	Sigma-Aldrich
NADPH	-	Sigma-Aldrich
NaH_2PO_4	p.a.	Sigma-Aldrich
Na_2HPO_4	p.a.	Sigma-Aldrich
NaOH	p.a.	Merck
NH_4Cl	p.a.	Merck

n-Hexane	p.a.	Merck
n-Propanol	p.a.	Merck
Nicotinic acid	p.a.	Sigma-Aldrich
Ninhydrin	p.a.	Merck
PAGERuler^{TM} Protein Ladder	-	Fermentas GmbH
Pantothenic acid	p.a.	Sigma-Aldrich
Paraffin oil	-	Sigma-Aldrich
Paraquat	-	Sigma-Aldrich
Peptone from caseine	for microbiology	Merck
Phenazine methosulfate	-	Sigma-Aldrich
Phosphoric acid	HPLC grade	Sigma-Aldrich
p-Nitrophenol	spec. grade	Sigma-Aldrich
p-Nitrophenolacetate	-	Sigma-Aldrich
p-Nitrophenolbutyrate	-	Sigma-Aldrich
p-Nitrophenolcaprylate	-	Sigma-Aldrich
p-Nitrophenyl arabinofuranoside	-	Sigma-Aldrich
p-Nitrophenyl xylopyranoside	-	Sigma-Aldrich
Pyridoxal 5'-phosphate	-	Sigma-Aldrich
Primer	-	MWG-Biotech AG
1-propanol	> 99.5%	Carl Roth GmbH
Proteinase K	-	Roche diagnostics
Pyridoxal-HCl	p.a.	Sigma-Aldrich
Random hexadeoxynucleotides	-	Promega
Reverse transcriptase	-	Promega
Riboflavin	p.a.	Sigma-Aldrich
RNase H minus	-	Promega
RNAprotect	-	Qiagen GmbH
RNAse	-	Roche diagnostics
RQ1 RNase-free DNase	-	Promega
SDS	research grade	SERVA
Sodium acetate x 3 H_2O	p.a.	Merck
Sodium citrate	p.a.	Merck
Sodium gluconate	p.a.	Merck
Sodium phosphate	p.a.	Merck
Sucrose	HPLC-grade	Gerbu Biotechnik GmbH
Sulfuric acid	p.a.	Merck
T4 DNA Ligase	-	Fermentas GmbH
TEMED	p.a.	Merck
Thiamine HCl	-	Sigma-Aldrich
Thymine	p.a.	Sigma-Aldrich
Tris	ultra pure	MP Biomedicals
Tris base	ultra pure	ICN Biomedicals GMBH
Tris HCl	p.a.	Merck
Tween 80	-	Mallinckrodt Baker B.V.
Water	molecular biology grade	Sigma-Aldrich
Wheat arabinoxylan insoluble	~ 80%	Megazyme
Wheat arabinoxylan soluble	> 95%	Megazyme
Wort	pre-stage beer process	Weihenstephaner
Xylan	-	Megazyme
Xylose	-	Sigma-Aldrich

Yeast extract	for microbiology	Merck

5.1.5 Restriction enzymes

All restriction enzymes used in this work were provided by MBI Fermentas GmbH, St. Leon-Rot, Germany and applied as recommended in manufacturer's instructions. Sometimes Fast Digest enzymes were used to reduce the incubation times.

5.1.6 Plasmids

As mentioned in chapter 3, heterologously expression of enzymes was achieved with the help of plasmid pBADB/*Myc*-His B (Invitrogen). The pBAD/*Myc*-HisB plasmid is a pBR322-derived expression vector designed for regulated, dose-dependent recombinant protein expression and purification in *E. coli*. Optimum levels of soluble, recombinant protein are possible using the *ara*BAD promoter (P_{BAD}) from *E. coli*. The regulatory protein, AraC, is provided on the pBAD/*Myc*-His vectors allowing regulation of P_{BAD}. In the presence of L-arabinose, expression from P_{BAD} is turned on while the absence of L-arabinose produces very low levels of transcription from P_{BAD}.

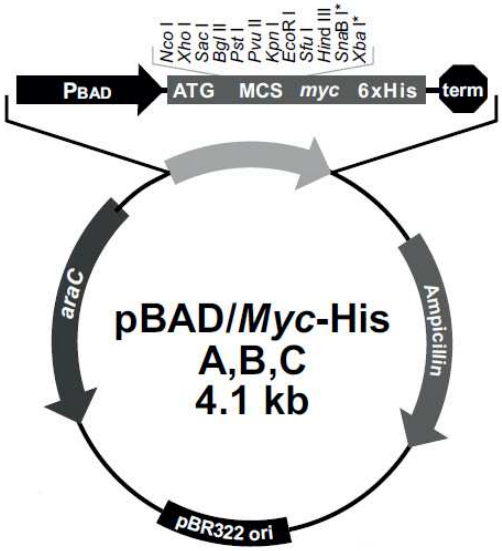

Figure 48. The figure summarizes the features of the pBAD/Myc-His vector, for more information see also www.invitrogen.com.

The plasmid pME-1 (see chapter 2 and 4) was transformed in *E. coli* DH5α and used for the construction of the integration vector pME-1Δ*gshR* and pME-1Δ*nox*, respectively. The plasmid PNG201 was transformed in *L. reuteri* BR11 (formerly *L. fermentum* BR11) according to Turner et al. (see chapter 4) to obtain *L. reuteri* BR11Δ*cyuC* defective in the L-cystine binding protein CyuC.

5.1.7 Media

Lactobacilli listed in chapter 2 and 3 were cultivated in modified MRS (mMRS) medium according to Stolz et al. (1995).

Table 27. Composition of mMRS-medium

Reagent	**Quantity**
Maltose	10.0 g
Fructose	5.0 g
Glucose	5.0 g
Peptone from casein	10.0 g
Yeast extract	5.0 g
Meat extract	5.0 g
K_2HPO_4 x $3H_2O$	4.0 g
KH_2PO_4	2.6 g
NH_4Cl	3.0 g
L-Cystein-HCl	0.5 g
Tween 80	1.0 g
Mg^{2+}-Mn^{2+}-stock solution	1.0 ml
Vitamin-Mix-stock solution	1.0 ml
MQ water	ad 1.0 L
pH	6.2

For agar plates, 1.5% agar was additionally added. The components were dissolved in 800 ml MQ water; all sugars were dissolved separately in 200 ml MQ water. Sugars and the other components were autoclaved separately, and mixed together after cooling. Finally, 1 ml sterile filtrated Mg^{2+}-Mn^{2+}-stock solution ($MgSO_4$ x 7 H_2O, 100 g/L; and $MnSO_4$ x 4 H_2O, 50 g/L) and 1 ml vitamin-mix-stock solution (0.2 g/L cobalamine, 0.2 g/L folic acid, 0.2 g/L nicotinic acid, 0.2 g/L panthotenic acid, 0.2 g/L pyridoxal-HCl, and 0.2 g/L thiamine) were added.

All *E. coli* strains (e.g. TOP10, DH5α) were cultivated using LB broth (peptone from casein 10 g/L, yeast extract 5 g/L, NaCl 5 g/L, MQ water ad 1 L, pH-value of 7.2) or LB agar plates, additionally 1.5% agar was supplemented.

5.1.8 Preparation of electro competent *E. coli* cells and transformation approach

200 ml LB broth in an Erlenmeyer flask was inoculated 1% with an overnight grown pre-culture of *E. coli*. The cells were incubated on a horizontal shaker 250 U/min at 37°C, and grown to an optical density (OD 590_{nm}) of 0.5–0.6. Following, the cells were harvested by centrifugation (4°C, 6000 U/min, 15 minutes) and washed twice with 200 ml sterile, ice-cold MQ water and once with 30 ml glycerol (10% (v/v)). Subsequently, the washed cells were resuspended in 1 ml glycerol (10% (v/v)), aliquoted to 100 µl and deep-frozen in an ethanol bath (-80°C) or used directly in transformations using electroporation. The transformations

were conducted with a Gene Pulser using appropriate cuvettes. 100 µl electro competent cells were thawed and mixed together with 2 µl ligation mix, then transferred to pre-cooled cuvettes. After transformation, 900 µl SOC broth (yeast extract 5 g/L, tryptone 20 g/L, NaCl 0.6 g/L, potassium chloride 0.2 g/L, magnesium chloride 10 mM, magnesium sulphate 10 mM, and glucose 20 mM) was instantly added to the cells; the cells were incubated at 37°C and 220 U/min for one hour. Subsequently, the cells were streaked out on LB agar plates supplemented with ampicillin and incubated aerobically overnight at 37°C. Grown transformants were picked with sterile toothpicks and again struck out on LB agar plates supplemented with ampicillin. The cloning success was assured with colony PCR using specific primer sets (forward primer: derived from the cloned gene; reverse primer: derived from vector pBADB/*Myc*-His B). Positive clones were incubated in 25 ml LB broth supplemented with ampicillin, harvested, and cryo cultured.

5.1.9 Determination of protein concentrations and performance of SDS-PAGE gels

The Bio-Rad protein assay, based on the method of Bradford was used for determining protein concentrations. One dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to evaluate protein size distribution in crude cellular extracts as well as purified fractions after FPLC. The 12% polyacrylamide gels were prepared as follows. The separating gel contained 3.35 ml MQ water, 2.5 ml Tris-HCl (1.5 M, pH 8.8), 40 µl of 25% SDS solution and 4 ml of acrylamide/bis 30%. The stacking gel consisted of 3.05 ml MQ water, 1.25 ml Tris-HCl (0.5 M, pH 6.8), 20 µl of 25% SDS solution and 665 µl of acrylamide/bis 30%. The cross-linking of the gels was started after supplementation of 50 µl ammonium persulfate (APS) 10% and 12 µl TEMED. After gel formation, 10 µl of sample was mixed with 10 µl of denaturating buffer and heated for five minutes at 94°C.

Table 28. Composition of denaturating and electrophoresis buffer

Denaturating buffer		**Electrophoresis buffer**	
Reagent	**Quantity**	**Reagent**	**Quantity**
Tris-HCl 0.5 M pH 6.8	1.0 ml	Tris base	9.0 g
Glycerol 87%	920 µl	Glycine	43.2 g
SDS 25%	700 µl	SDS	3.0 g
2-Mercaptoethanol	400 µl	MQ water	600 ml
Bromphenol blue	400 µl	pH	8.3
MQ water	4.58 ml		

10 µl of denatured protein fractions were applied to the gel cavities; for protein size determination 10 µl PAGERuler^{TM} protein ladder was used. Separation was started at 70 V for 15 minutes, and then voltage was increased to 120 V for approx. two hours. Proteins were visualized by Coomassie staining method. Following, the gels were immersed in staining solution (filtrated mixture of 80 ml MQ water, 120 ml ethanol, 100 ml acetic acid (20%), and one tablet PhatGel^{TM}BlueR) and heated in a microwave for one minute. Subsequently, the gels were gently shaken for at least 10 minutes. Staining solution was discarded and a de-staining solution (40 ml ethanol, 20 ml acetic acid (100%), 140 ml water) was applied and heated again in the microwave for approximately one minute. The de-staining solution was exchanged and the gels were shaken for 3-4 hours. Finally, the gels were washed with MQ water and digitalized by scanning.

5.1.10 PCR and agarose gel electrophoresis

Generally, DNA was amplified with thermo cyclers (PRIMUS 96 plus) by using Taq Core polymerase. Unless stated otherwise, 1.5 mM $MgCl_2$, 0.2 mM dTNPs, 0.5 µM primers, water, and 1.5 U Taq polymerase were used for PCR. Standard conditions for PCR were as follows: Initially, genomic DNA was denatured (94°C/3 min). Following, 32 cycles were performed consisting of a denaturation step (94°C/1 min), a primer annealing step (melting temperature of primer minus 3°C/45 seconds), and an elongation step (72°C, depending on fragment length – extension rate of the polymerase, 1 kb/minute). PCR's for cloning or sequencing purposes were performed with a proof-reading polymerase (KOD Hot Start DNA polymerase). 1.5 mM MgSO4, 0.2 mM of each dNTP, 0.3 µM of primers, water, and 0.02 U/ml KOD polymerase was used in PCR. Standard conditions for PCR were as follows: Initially, genomic DNA was denatured (94°C/20 seconds). Following, 32 cycles were performed consisted of a denaturation step (94°C/20 seconds), a primer annealing step (lowest primer Tm°C for 15 seconds), and an elongation step (70°C, depending on fragment length – extension rate of KOD, 1 kb/15 seconds for targets of 500–1000 bp).

Agarose gel electrophoresis was performed to evaluate the amplification success. 1–1.5% agarose gels were prepared with 0.5 x TBE buffer (10 x TBE buffer: 150 g/L Tris, 26.2 g/L boric acid, 9.0 g/L EDTA, pH-value of 8.9) or 1 x TAE buffer (50 x TAE buffer: 0.1 M EDTA, 1 M acetic acid (100%), 2 M Tris, pH-value of 8.2), respectively. PCR products were mixed with a loading dye in a ration of 5:1. Separation was routinely performed in electrophoresis chambers (120 x 138 mm) at 90–120 V for one hour. DNA ladders were applied and served as a control. Gels were stained with ethidium bromide; the DNA was

visualized under UV-light (wavelength 320 nm) and finally digitalized by gel documentation system.

5.1.11 Isolation of genomic DNA, plasmids, and purification of PCR products

PCR products obtained in chapter 2, 3, and 4 were purified using the QIAquick PCR purification kit; for preparative TAE agarose gels the isolation was achieved by using the PeqGold gel extraction kit. Plasmids were isolated with the help of PegGold plasmid miniprep kit or Wizard Plus SV Minipreps DNA kit according to the manufacturer's instructions. Chromosomal DNA of lactobacilli for screening studies was isolated using the E.Z.N.A. bacterial DNA kit. In general, the evaluation of the isolation success was determined by analytical agarose gel electrophoresis. Moreover, the amount of DNA was quantified by analysis with a Nanodrop 1000 device according to the manufacturer's instructions.

5.1.12 Digestion and ligation

Restriction enzyme digestions with the appropriate enzymes and ligations with T4-DNA ligase were performed according to the recommendations of the manufacturer (Fermentas).

5.2 SEQUENCES

The nucleotide sequence of the *Lactobacillus sanfranciscensis* TMW1.53 glutathione reductase (*gshR*) and CyuC-like protein genes have been assigned the GeneBank accession numbers DQ866807 and EF422159, respectively. The sequence of the genomic region of *nox* has been registered under the accession number AB035801. The nucleotide sequences of the *Lactobacillus sanfranciscensis* TMW1.53 predicted Mn^{2+}/Fe^{2+} transporters of the nramp family MntH1 and MntH2 protein genes have been assigned with accession number FJ440569 and FJ440570, respectively. The genes of the heterologously expressed cinnamoyl esterases of lactobacilli used in chapter 3 possess the following accession numbers: BAG26088 for *Lactobacillus reuteri* TMW1.106, BAG27654 for *Lactobacillus fermentum* TMW1.890, CAD65143.1 for *Lactobacillus plantarum* TMW1.460, ABJ61050 for *Lactobacillus gasseri* TMW1.1173, AAV43644 for *Lactobacillus acidophilus* TMW1.697, and ABX27728 for *Lactobacillus helveticus* TMW1.1176. The cloned genes of *Lactobacillus brevis* TMW1.1326 for α-L-arabinofuranosidase protein and β- xylosidase protein possess the accession numbers YP_796302 and YP_794569, respectively.

Sequence of the **cystathionine-γ-lyase** of *Lactobacillus fermentum* TMW1.890:

ATGAAGTTTAACACCCAATTAATTCACGGCGGCATTAGCGAAGACCCAACCACCGGGGCGGTTTCGACCCCAA
TTTACCGCTCCTCAACCTTCCGTCAACACGTCCTCGGCGGTGGCCCCAAGTGGGAATACGCCCGGACCGGCAAC
CCGACCCGGGCCTCCTTGGAACTCTTAATGGCCCAACTAGAAGAAGGGGTTGCTGGCTTCGCCTTTGCCTCGGG
ATCGGCCGCCATCCACGCCGTCTTTTCCCTCTACTCCTCCGGCGATCACTTCATCATTGGTAAGGACGTCTACGG
GGGGACCTTCCGTTTGATCAACAAGGTCCTGAAGCGTTTCGGCCTCGAATTTACGGTTGTCGACACCCAGGACC
TAGCGGCGGTCGAAGCGGCCGTGCAAGACAACACGGTGGCCATTTACTTTGAAACACCCACCAACCCGCTCTT
GGAGGTTTCAGACATCGAAGCGATTGCTAAGATCGCCAAGAAGCATGGCTTAAAGACGGTCATTGATAACACC
TTTGCCACGCCGTATAACCAACGCCCGCTGACCCTGGGCGCCGACATTGTAGTTCACTCTGCCACCAAGTACCT
GGGCGGTCACTCCGACGTGGTGGGGGGGATTGCCGTTACGAACGACGAGGAAATTGCCGAACAGTTGGCCTTC
ATCCAAAACTCGATTGGCGCCGTCCTTGGGCCCGACGACTCCTGGCTTCTGATGCGGGGGATTAAGACCCTCGG
CGCCCGAATGCGGATCCACCATGAAAACACCGCCGCCGTCATTGAACTGTTGGAAAAGGACCCGCGGGTTGCT
CGGGTACTGTACCCGGGGCTGCCAGATTTCCCGGGACATGATATCGCGGCTAAGCAAATGGACCACTTCGGCG
CCATGGTGTCCTTTGAACTGCAAACGGGGCTCTCGGCAAAGAAGTTTGTCGAAAGCCTGCAGGTGATTACCCT
AGCCGAAAGCCTCGGTGGGATCGAGAGCCTGATTGAAGTGCCGGCGGTGATGACCCACGGTTCCATTCCGCGT
GAGGTCCGCCTGCAAAACGGGATTAAGGACGAATTGATCCGCTTGTCGGTTGGGATCGAAGACGAAGAAGAC
CTCGTGGCAGACTTACAGCAAGCCCTGGACCAATTATAG

MKFNTQLIHGGISEDPTTGAVSTPIYRSSTFRQHVLGGGPKWEYARTGNPTRASLELLMAQLEEGVAGFAFASGSAA
IHAVFSLYSSGDHFIIGKDVYGGTFRLINKVLKRFGLEFTVVDTQDLAAVEAAVQDNTVAIYFETPTNPLLEVSDIEAI
AKIAKKHGLKTVIDNTFATPYNQRPLTLGADIVVHSATKYLGGHSDVVGGIAVTNDEEIAEQLAFIQNSIGAVLGPD
DSWLLMRGIKTLGARMRIHHENTAAVIELLEKDPRVARVLYPGLPDFPGHDIAAKQMDHFGAMVSFELQTGLSAK
KFVESLQVITLAESLGGIESLIEVPAVMTHGSIPREVRLQNGIKDELIRLSVGIEDEEDLVADLQQALDQL

Sequences of *L. sanfranciscensis* TMW1.53 related to genes involved in the oxidative stress response (see also chapter 4.3.8):

Glutaredoxin-like NrdH (accession number: ABK51755)

…MNSITVFTKNNCIQCKMTKRFLEEHNIDFVEKNTSENPEFVTYLKELGFQVVPVVEVEGAESFTGFRPDCLNKLVA
EFA

Thioredoxin (accession number: ABK51756)

…MTIVDFWAPWCGPCKIMDPILDKLEQQFAGKVKFAKVNVDNQQEIAKEYSVYGMPTFVLFKNGKGVEKVTGVY
PIEKLTHYLNRKLEEVENSNG

Thioredoxin reductase (accession number: ABK51757)

MSRKLYDITIIGGGPIGMFAGFYAGMRNAKVQIIESLSELGGQVNALYPEKTILDVAGFAGLKGSELINNLQTQLDTM
PEVEQRVGTKVTNVIRKDDHFEIETDQATYETKAVILATGNGSFKPRELRADNVDAVAEKFITYSVRDLKKFANQD
VIVAGGGDSAVDMALMLEPVANHVSLLHRRNEFRGLENMVDKLKASSVEILTPYLIKQLDEIDGRLQVTAKKNEFK
LISS....

Nach Submission vervollständigt zu:

MSHKLYDITIIGGGPIGMFAGFYAGMRNAKVQIIESLSELGGQVNALYPEKTILDVAGFAGLKGSELINNLQTQLDT
MPEVEQRVGTKVTNVIRKDDHFEIETDQATYETKAVILATGNGSFKPRELRADNVDAVAEKFITYSVRDLKKFANQ
DVIVAGGGDSAVDMALMLEPVANHVSLLHRRNEFRGLENMVDKLKASSVEILTPYLIKQLDEIDGRLQVTAKKLKT
DDESTFMADKLVVNYGFISNNKDLESWQIQPALNHHLVEVNSEMETSEPGVFSIGDQATYPGKDTLIVTGFGEAPVA
INEIMKRLYPDRRMPIHSTALHK

Ribonucleotide reduction protein NrdI

MKPLKLIFISNTGNTRNFAENLVNYAQEQNQKNPDYPLLSIEEISEQTDFGNETEPYFVSVPTYLSGGDGTGDNVKEV
MTTILGEYLDYHDNAKQCLGIIGSGNKNFNIQYCLTAKRYSKRFNVPFLADFELRGNDKDVQRIYEDMVERTKEVN
Q

Ribonucleotide reduction protein NrdH (rot), NrdE (grün), NrdF (blau)

AAAACTCGTGATCCATAGTCAATGGTGCTTTTAGAAACACACCGTTATCTGAAACAAAACTAAGCGTGTAATTCAATAAGGTATATTCCCAATG
TTTTTCATCATGTTGAACATCTGGATTTTCTGTGTAATAAAAGTTGTTCATGATAGCACGTCCTCAAAAATATTTTTTAGTTTCACACACTATA
TATAGCGTGTTTCTCAAGTGCACACTCCATATATGGTGTGTTTTCATTTGAAAGATTAAGTGTTCGTAGTAAAATCTATT
ATATCGTATAAACAGGAGATGAAGATTATGAATTCAATTACAGTATTTACTAAAAACAATTGTATCCAATGTAAAATGACTAAGCGTTTCCTTG
AGGAACACAACATCGATTTCGTAGAAAAAAACACTTCTGAAAACCCAGAATTTGTTACCTACTTAAAAGAGTTAGGTTTCCAAGTAGTTCCAGT
TGTTGAAGTCGAAGGTGCCGAATCATTTACTGGTTTTAGACCTGATTGCCTAAACAAACTAGTAGCAGAATTCGCTTAA
CATTATATTACCTATTATACACAACAAGGAGCACTAGGAAT
ATGTCGCTAAAAGACCAAACTGATGTTAGTTATTATAGATTAAATAACGAGATCAACATCCCATCAAAAGATGGAAAAATTAGACTAGAAAAAG
ATAAAGAAGCCCTAGATGCTTTTATTAAAGAAAACGTGATTCCTAATACCGTAAAGTTTGATTCTTTAAAGGATCGTTTGGATTACTTAACTAA
AAATAACTACATTGAAGCTAGCTTTTTAGAAAAGTATCCTTTTTCATTTATTGAAAAACTTTACCAATACTTAGAGGATCAACATTTTACCTTT
AAATCATTTATGGCTGCCTATAAATTTTATGCACAATATGCCTTAAAAACAAATGATGGTAATCAATATCTTGAAAGATACATTGATCGGACTG
CAATGAACGCCCTCTTTTTAGCTGACGGTGATCAAGAATTAGCAATGCGTTTAGCTGATGAATTAATTCACCAACGTTTCCAACCAGCTACGCC
AACTTTCTTAAACGCTGGAAAAAAACGGCGTGGAGAATTAATTTCATGTTTCTTAATCCAAACTACTGATGATATGAATAGTATCGGAAGAACC
ATTAATTCGGCCTTACAACTCTCTAAAATTGGTGGAGGTGTAGGAATTAACCTTTCTAACCTTCGTGCTGCTGGAGATCCAATTAAGCACATTC
AAGGCGCTGCTAGTGGAGTAATGCCAGTCATGAAACTGTTAGAAGACAGTTTTACCTACTCAAATCAATTAGGCCAACGTCAAGGTGCTGGTGT
TGTTTATCTTAGTGTCTTTCATCCTGATATTATTGACTTTCTTTCAGTTAAAAAAGAAAATGCTGATGAAAAAATTCGAGTTAATACCTTATCT
TTAGGACTAACCGTTCCTGATAAGTTTTATGAACTTGCTAAAAATGATCAAGATATGTATCTCTTTAGCCCTTATGATGTGGAAAAAGAATATG
GAGTTCCATTCTCTTACGTTGACATCACTAAAGAATATGACAATTTAGTTGCTAACGAAAACATCCGCAAGAAAAAAATGAAGGCTCGTGATTT
AGAAACCGAAATCGGTAAATTACAACAAGAATCCGGTTATCCTTACATCATGAACATTGATACTGCTAACCGGGAAAAACCAGTTGCCGGTAAA
ATTGTCATGAGTAACTTGTGTTCTGAAATTATGCAAGTGCAAACCCCATCAATCATTAATGACTTGCAACAGTATGACCAATTAGGGACTGATA
TTTCATGTAACCTAGGTTCAACAAACGTCGTTAATTTGATGAAATCAGATGATTTTGGTGAATCTGTTAACACAATGGTGCGTGCCTTAACCTA
CGTGACCGATGAAAGTAAAATTGATGTTGTTCCTTCAATTAGAAGAGGAAATGATTTAAGCCATTCGATTGGACTTGGTGCCATGGGTCTTCAC
TCATTTCTCGCTTTAAATCATATGTATTACGGTTCGCCTGAATCAATCGAATTTACTGGTGTTTACTTTATGCTCTTGAACTACTGGACTCTAG
TTGCATCTAACAAGATTGCTAAAGAACGCGGTAAGTCATTCCATAACTTTGAAAACAGTAAATATGCAGATGGTACTTATTTTGATAAGTACAC
CGCCAAAGATTGGGGTCCCCAATCAGAAAAAGTTAAGGACCTCTTCAAGGGTACCTTTGTGCCTTCTGAAAAAGACTGGGAAGAGCTTAAGAAG
TCAGTTATGACTTATGGTTTATATCATCAAAACCGGATGGCAGTTGCTCCTAATGGTTCAACAGCCTACATTGGTGACGCTACCGCAAGTATTG
CCCCAATCGTTAGTCGAATTGAAGAACGACAAGATGCTAAAATTGGAACAATTTTTTATCCCGTTCCTTACCTTTCAAACGATACCCTTCCATA
TTATGAATCAGCTTATGATATTGATATGCGTAAAGAAATCGATATCTTTGCTGAAGCCCAAAAACACGTTGATCAAAGTTTAAGTATGACCCTC
TTCATGCGTTCTACCATTCCTGAAGGGATGTACGAATGGAAAAATGGTCGTGATCCAAAGATGTCAACACGTGATTTAACAATCTTAAGAAATT
ATGCTTACCATAAAGGCATTAAATCAATTTACTACGTTAGAACTTTCACTGATAACAACGACACAGTTGGTGCTAACGAATGTCAAAGCTGTGT
CATTTAG
GAGGATTATATCA
ATGAATATGAACGAAATTTTAACGGGTAAGTTAAAGGGTAATTACAAAGCCATTAACTGGAATCGAGTTGATGACGATGTTGATGAAGCAACCT
GGCACAAACTTACAGAACAATTTTGGTTAGATACCCGAGTACCAGTTTCTAACGATTTAAAAGATTGGCGTGAACTTGATGACGATCACAAATG
GCTCGTTGGACATGTCTTTGGTGGATTAACTTTATTAGATACCCTTCAATCTCAAGATGGAATGGCATCATTAAGAGCAGATGCACATACTCAA
CATGAAGTTGCGGTATTAAACAATATTCAATTCATGGAATCAGTCCATGCTAAAAGCTATTCTACAATCTTTTCAACTTTAAACACTCCTAAAG
AAATCAATGAAATTTTTGACTGGAGTGATTCTGAAGAATTTTTACAAAGCAAAACCAAAAGAATTTATAACCTTTATCATAATGATGAACACCC
ATTAAAAAAGAAAATTTCAAGTGTATTTCTAGAAACCTTTCTCTTTTACTCTGGATTCTTCACCCCACTTTGGTATCTAGGTCATAATAAATTG
ACCAATGTGGCTGAAATCATCAAGTTAATTATTCGGGATGAATCAGTTCACGGTACCTATGTTGGTTACAAATTTCAGATTCAATTTAATCAAT
TAAGTCCTAATGAACAAGCTGAACTTAAAGATTGGATGTATAACTTCCTTTATGACTTATACGAAAACGAGGTTAATTACACCCATCTTTTATA
TGATAAAACTGGTTTAACAGATAAAGTGCTTACTTTCATCCGTTACAATGCCAATAAAGCTTTAATGAACTTGGGTCAAGACCCAATGTTTCCA
GATACTGCTGAAGATGTTAACCCCGTTGTGATGAACGGAATTTCAACTTCAACAGTTAATCATGACTTCTTCTCAGAAGTTGGAAATGGTTATC
GTCTTGGTAATGTTGAAGCAATGTCAGATGATGATTATCTATTTGGTTCTTGGGAAGATAAACACAAAAATAAATAA

ATTTTAAATAGTAAAAGAGCCACTTCAATGAAGTGGCTCTTTTTTTAAACTATTTTTCACTATATTTCTTAAAATTAGTTTTAATAAAATCAGC
TGGAATGTTTTTTAAATCAACTGCGTTAATC

NADH peroxidase

ATGAAAGTAGCAGTTGTAGGTTCATCACACGGTGGTTTTGAAACTGTAAGAGGAGTATTACACGATTTTCCCAA
TGCTGAGATTGATTGGTATGAAAAGGGCGATTTTGTTTCTTTATTATCATGTGGAATTGAATTGTATCTTCAAGG
AGTCGTTAAGGATGTTAATTCCGTTAGTTATGCAACAATTGGTGGAATGGAAGCAAAGGGCGTTCATGTTTATA
TTAATTCTGAAGTGACTAGCATTGACCCAGAACAACATTCAATTAAGGTTGTTGATGTTAATAACGGTGAAGA
AACTGAAAGTAAATATGATAAGTTAGTTCTTTCGTTAGGAGCGGTTCCTTTTGAACTACCTGTGCCAGGAAAAG
ATTTAAAGAATATTTATGCTATGCGTGGATGTGACTGGGAAATTCTTTTAAAGAAAGCTGAAGTTGACCCTGAT
ATTAATAATGTCTCAGTCATTGGTTCAGGTTATATTGGAATTGAAGCCGCCGAATCATTTGCAAAGGCCGGTAA
GAAAGTTACTATTATTGATCAAAATCCAACTATTTTAGGAACTTATTTAGATTCAGAATTTACAGATATCTTAA
CTAAAACGCTTGAAGATCACGGTATTCAATCCGTTAAAGAATTTATTGGTAATGATGAAAATAAAGTAACCAG
TTTGGTAACGACAACTGGTGAAACTATTCCTACTGATTTAGTGATTGAAGCTGCAGGAATTTGTCCAGCAACTG
AATGGTTGAAAGATACTGTTAAACTTGATAGCCAAGGCTTAATTATGACTGATGAATATCAAGAAACTAGTCA
ACCAGATATTTTTGCCGTTGGTGATGCCACTAAAATTGAGTTTGCCCCAACTGGAACCAAAAAATTAATTGCGC
TTGCACCAAATGCTCGTCGACAAGGTCGTTCAGCTGCTTATAATTTAAATGAAAAACGTCGCAAAACAACTGCT
GTCTCTGGATCATCTGCACTTCATGTCTATAATTACAAGTTTGCTTCAACTGGATTAAAAGATGTTACCGCTAA
AAAAATGGGTATTGACGTTGAATCAGTATTTTTAACTGATGATAAAGTACCAGCTTTTGTACCAGCTTCAAACA
ATGCAAAAGTATACTTCAAATTAACTTTTGATCCGCGTACTCGTGAAGTTTTAGGGGCTCAAATTATGTCTAAA
CAAGATGTAACGGCTAATATCAATGCAATTTCGCTAGCAATCCAAAAACACATGACAGTTGATGAATTGGCTT
ATGCTGATTTCTTCTTCCAACCAGGCTTTGACCGTCCTTGGAACGTTATGAATATTGCAGCACAAAAAGCACAA
GATAA

6

6. ABBREVIATIONS

4VG	4-vinylguiacol
4VP	4-vinylphenol
A.	Aspergillus
A	arabinose
aa	amino acids
approx.	approximately
APS	Ammonium persulfate
ara-2	α-L-arabinofuranosidase (heterologous expressed)
Asp	aspartic acid
ATP	Adenosine-5'-triphosphate
AX	arabinoxylan
B.	Bacillus
bp	base pairs
BSA	bovine serum albumine
BspA	basic surface protein
C.	Clostridium
C	carbon
cDNA	copy DNA
cfu	colony forming unit
CO_2	carbon dioxide
DFT	dehydro-ferulic acid-tyrosine
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
CgL	cystathionine-γ-lyase
DBM	dinucleotide-binding motifs
DMSO	dimethyl sulfoxide
DNA	desoxyribo nucleic acid
dNTP	desoxy nucleotide triphosphate
E.	Escherichia
E.C.	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
EF	ethyl ferulate
e.g.	for example
FA	ferulic acid
FAD	Flavin adenine dinucleotide
FAN	free amino nitrogen
Fe	iron
FD	fast digest
FPLC	fast protein liquid chromatography

FSI	Forschungsstelle 1 Garching
GC/MS	Gas chromatography–mass spectrometry
Gln	glutamine
Gly	glycine
GMP	glutenin macropolymer
GS	glutenin subunits
GSH	glutathione (reduced)
GshR	glutathione reductase
GSSG	glutathione (oxidized)
H_2O	water
H_2O_2	hydrogen peroxide
HCL	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFA	hydroferulic acid
His	histidine
HMW	high molecular weight
HPLC	high performance liquid chromatography
IPTG	Isopropyl-β-D-thiogalacto pyranoside
L.	Lactobacillus
LAB	Lactic acid bacteria
LB	lysogeny broth
LMW	low molecular weight
MAP	mucus adhesion protein
MES	2-(*N*-morpholino)ethanesulfonic acid
Mg	magnesium
MRS	de Man, Rogosa and Sharpe
mMRS	modified MRS medium
Mn	manganese
MTBE	Methyl-tert-butylether
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Mw	molecular weight
N_2	nitrogen
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
n.d.	not detected
No.	Number
Nox	NADH oxidase
NSP	non-starch polysaccharides
nt	nucleotide
O_2	oxygen
OD	optical density
PAGE	polyacrylamide gel electrophoresis
p-CA	coumaric acid
PCR	polymerase chain reaction
PDA	phenolic acid decarboxylase
PDC	*para*-coumaric acid decarboxylase

PLP	pyridoxal 5'-phosphate
PMS	phenazine methosulfate
p-NPA or *p*-NP-C2	*para*-nitrophenyl acetate
p-NP-ara	*para*-nitrophenyl arabinofuranoside
p-NP-C4	*para*-nitrophenyl butyrate
p-NP-C8	*para*-nitrophenyl caprylate
p-NP-xyl	*para*-nitrophenyl xylopyranoside
RAPD	random amplification of polymorphic DNA
RNA	Ribonucleic acid
SDS	sodium dodecyl sulphate
Ser	serine
SOC	super optimal broth + glucose
SOD	superoxide dismutase
TA	dough yield
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TEMED	Tetramethylethylenediamine
TLC	Thin layer chromatography
Tm	melting temperature
TMW	Technische Mikrobiologie Weihenstephan
TPC	Total plate count
Tris	Tris-(hydroxymethyl-) aminomethane
Trx	thioredoxin
TrxR	thioredoxin reductase
Tyr	tyrosine
U	Units
UV	ultraviolet
v	Volume
W.	Weissella
WE-AX	water extractable arabinoxylan
WU-AX	water unextractable arabinoxylan
X	xylose
xyl-1	xylosidase (heterologous expressed)

Table 29. One letter code for nucleotides

A	ATP
T	TTP
G	GTP
C	CTP
M	A/C
R	A/G
W	A/T
S	G/C
Y	C/T
K	G/T
H	A/C/T
D	A/G/T
N	A/T/G/C

Table 30. One letter code for amino acids

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine
X	any amino acid

Table 31. Units of measurements

µl, ml, l	microliter, milliliter, liter
nm, µm, mm, cm	nanometer, micrometer, millimeter, centimeter
nmol, µmol	nanomol, micromol
µM, mM, M	micromolar, millimolar, molar
ppm	parts per million
s, min, h	second, minute(s), hour
mbar, bar	millibar, 100.000 Pascal
µg, mg, g	microgramme, milligramme, gramme
U	Unit
rpm	rounds per minute
°C, °P	degree Celsius, degree Plato
kV	kilo Volt
µF	micro Farad
g	gravitational acceleration
kDa	kilo Dalton
%	percentage