Conversion of Protein to Bioactive Peptides in Sourdough Fermentation

By

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Abstract

Sourdough fermentation can generate various peptides and amino acids to improve bread quality, such as taste, flavor and texture. The aim of this PhD dissertation was to investigate the synthesis mechanisms of bioactive and taste active peptides or amino acids during fermentation and their effect on food quality.

To understand the effect of glutamate on bread taste, sourdough was fermented with GABA-accumulating *L. reuteri* 100-23 and the glutamate-accumulating mutant *L. reuteri* 100-23ΔgadB. The difference of glutamate contents between sourdough breads was equal to its taste threshold (0.03%). Nevertheless, the taste difference between bread fermented with *L. reuteri* 100-23 and 100-23ΔgadB was attributed to umami. Besides, a consumer panel (n=40) ranked the salty taste of *L. reuteri* sourdough bread with 1.0% NaCl equal to that of the reference bread with 1.5% NaCl but higher than that of the reference bread with 1.0% NaCl. These results confirmed the umami taste of glutamate in sourdough bread and the taste enhancer activity of glutamate in bread.

A series of γ-glutamyl dipeptides with kokumi taste activity was first found in sourdough. Proteolysis and strain specificity are the key factors to generate dipeptides. Amino acids model of *Lactobacillus* confirmed the contribution of microorganisms in the generation of γ-glutamyl dipeptides, even though the specific metabolism pathway is still unclear. At subthreshold of γ-glutamyl dipeptides, the sourdough bread with γ-glutamyl dipeptides ranked higher in salty taste than regular bread and type I sourdough bread. Moreover, the significant difference in salty taste between sourdough breads fermented with *L. reuteri* LTH5448 and *L. reuteri* 100-23 is attributed to the significant difference in

the level of γ-glutamyl dipeptides. This result indicated the contribution of γ-glutamyl dipeptides to the taste of bread. The use of sourdough fermented with glutamate and kokumi peptide accumulating lactobacilli allowed reduction of NaCl without any adverse effect on the other taste or quality attributes.

The ACE-inhibitory tripeptides have been found during sourdough fermentation. The bread-making steps, including kneading, proofing and baking affect the stability of peptides. The levels of peptides after different thermal treatments, including steamed bread, baked bread and soda crackers, were compared. The X-PP was the most stable peptides during dough stage but decreased during baking whereas other ACE tripeptides remained stable during baking but decreased during the dough stage. The cumulative concentrations of 8 ACE-inhibitory peptides in steamed bread and baked bread likely meet *in vivo* active concentrations.

In conclusion, through the combination of glutamate, kokumi peptides, ACE-inhibitory peptides as well as GABA, new functional bread can be produced with low sodium and bioactive compounds without adverse effects on taste or other quality attributes.

Preface

This thesis is an original work by Cindy Jing Zhao.

Chapter 1 has been included in the manuscript Zhao CJ, Gänzle MG. Contribution of taste active amino acids and peptides to the fermented food. I collected and read the literature and wrote the manuscript with Dr. Gänzle.

Chapter 2 has been published as Zhao CJ, Kirnner M, Wismer W and Gänzle MG (2015). Effect of glutamate accumulation during sourdough fermentation with *Lactobacillus reuteri* on the taste of bread and sodium-reduced bread. Cereal Chem 92(2): 224-230. The study was designed by Zhao, Kirnner, Wismer and Gänzle. Trained panel and texture measurement was designed and executed with the collaboration with Kirnner in Switzerland. I compiled, designed and analysed the experimental data and wrote the manuscript with Dr. Gänzle.

Chapter 3 has been included in the manuscript Zhao CJ, Gänzle MG. Synthesis of γ-glutamyl dipeptides during sourdough fermentation by *L. reuteri*. I designed and performed the experiment and wrote the manuscript. Dr. Gänzle provide a lot of comments and suggestions to edit the manuscript.

Chapter 4 has been published as Zhao CJ, Hu Y, Schieber A and Gänzle MG (2013). Fate of ACE-inhibitory peptides during the bread-making process: quantification of peptides in sourdough, bread crumb, steamed bread and soda crackers. Journal of Cereal Sciences 57: 514-519. Hu established the LC-MS in MRM quantification method. I performed the experiment and wrote the manuscript.

Dedication

I would like to dedicate this PhD thesis to my beloved parents and my husband. Thanks for your love, support and help in my life.

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First and foremost I would like to express my sincerest gratitude to my supervisor, Dr. Michael Gänzle, who has supported me throughout my thesis with his patience and knowledge whilst allowing me the room to work in my own way. I attribute the level of my PhD degree to his encouragement and effort and without him this thesis, too, would not have been completed or written. He provided me with direction in my life and study, and became more of a mentor and friend, than a professor. It was though his, persistence, understanding and kindness that I completed my PhD degree. I doubt that I will ever be able to convey my appreciation fully, but I owe him my eternal gratitude.

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Finally, I thank my parents for supporting, mentoring, and guiding me throughout all my life. Also thanks to my husband to help me to format and edit this thesis. Without you, I can’t achieve it. This thesis is dedicated to you.

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List of abbreviations

ACE	Angiotensin converting enzyme
FAN	Free amino nitrogen
GABA	γ-Aminobutyrate
γ-GCS	γ-Glutamyl cysteine synthetase
GGT	γ-Glutamyl transferase
GS	Glutathione synthetase
GSH	Glutathione
HRM- qPCR	High resolution melt quantitative PCR
HPLC	High performance liquid chromatography
IC50	Inhibitory concentration inhibiting ACE activity by 50%
IMP	Inosine-5'-monophosphate
LAB	Lactic acid bacteria
MS	Mass spectra
MSG	Monosodium glutamate
MRM	Multiple reaction monitoring mode
NSLAB	Non starter lactic acid bacteria

1 Contribution of taste active amino acids and peptides to fermented food

1.1 Introduction

Food taste determines food selection, intake, absorption, and digestion and hence contributes greatly to consumer satisfaction (Barylko-Pikielna & Kostyra, 2007; Beksan et al., 2003). Six basic tastes, including salt, sweet, and umami, sour, bitter, and oleogustus, are detected through gustatory nerves or taste receptors, and processed by taste buds that are distributed across different papillae on the tongue and palate epillium (Running et al., 2015). Sweet, umami and bitter tastes are the most important taste attributes, which are related to food acceptance or rejection (Barylko-Pikielna & Kostyra, 2007). Attraction to sweet taste helped our early ancestors to identify carbohydrate rich foods and to ensure intake of energy (Behrens et al., 2011). Due to health concerns, such as obesity and type-2 diabetes, low calorie sweeteners are in high demand. Aspartame, saccharin, acesulfame K, sucralose and neotame are approved but continue to meet safety concerns (Behrens et al., 2011). Umami molecules, typically L-Glu, impart savory taste and increase other taste intensities (Jinap & Hajeb, 2010). 5'-Ribonucleotides including inosine-5'-monophosphate and guanosine-5'-monophosphate, also drastically elicit the umami taste and act synergistically with glutamate (Jinap & Hajeb, 2010). A limited level of bitterness in food may be desirable. However, since humans have an innate desire to reject bitter taste, excessive bitterness in food is a defect. Debittering can be achieved through enzymatic methods or use of a taste masker (FitzGerald & O'Cuinn, 2006).

Saltiness often determines the sensory acceptance of savory foods, such as soups, sauces, snacks, and bakery products (Schindler et al., 2011). However, salt intake in industrialized nations exceeds the amount recommend by WHO by 80-100%. Excessive salt intake is related to kidney, cardiovascular, and other chronic diseases. Sodium reduction, achieved through a partial replacement of sodium chloride with potassium chloride, a combination of different taste enhancers, such as glutamate, peptides or modified physical properties of food, has been investigated (Blesa et al., 2008; Schindler et al., 2011; Zhao et al., 2015). A series of γ-glutamyl dipeptides, named as "kokumi", isolated from edible beans and Gouda cheese, enhance other taste attributes, but they do not have a taste on their own (Toelstede & Hofmann, 2008b; Toelstede et al., 2009; Toelstede & Hofmann, 2009). The kokumi taste impression is described as mouthfulness, complexity, continuity, and long lasting taste.

Food fermentation is one of the oldest preservation methods. It can also increase aroma and enhance palatability. However, few researches have studied the taste of fermented food. Therefore, this review will focus on the taste of fermented food products. Soy sauce, cheese, meat products, and bread are discussed in consideration of the fermentation procedure, and cross-section of raw materials and organisms. The changes in ham and fermented sausages are parallel; therefore dry fermented sausage and ham will be discussed in this review. This review will compare and summarize the generation and contribution of taste active amino acids and peptides to fermented foods. In addition, the effect of amino acids and peptides to salt reduction and the debittering of food will be discussed.

Soy sauce, cheese, fermented meat, and bread are major non-alcoholic fermented foods from cereals, meat and dairy, which are widely consumed and well studied. Soy

sauce is used widely to impart salty, savory, and rich taste to food (Oka & Nagata, 1974a). In koji fermentation, enzymes from *Aspergillus* hydrolyze cereal proteins. In moromi fermentation, yeast and LAB decrease the pH and produce alcohol (Lioe et al., 2010). The final product (moromi) contains high concentrations of nitrogen compounds, including 40-50% free amino acids and remaining small peptides. Cheese is widely consumed due to its special taste, flavor, and texture. It is made from milk that is cultured and heated until curds separate from whey. During cheese ripening, molds or LAB generate distinct tastes (Toelstede & Hofmann, 2008b). Similarly, surface molds or LAB on fermented sausage contribute to a typical flavor, odor, and texture of the product (Ordonez et al., 1999). Sourdough has been used as baking improver for a long time. During sourdough fermentation, cereal enzymes and LAB generate free amino acids, fatty acid, EPS, acetic acid, and decreased pH, which enhance bread taste and texture (Gobbetti& Gänzle, 2007; Gänzle et al., 2007; Gänzle et al., 2008).

1.2 Generation of taste active amino acids and peptides during food fermentation

Taste compounds can be generated through the primary proteolysis of raw material by proteases from endogenous enzymes or microorganisms (Figure 1-1). Microorganisms release small peptides or free amino acids during secondary proteolysis (Table 1-1, Table 1-2). Free amino acids are converted into derivatives or new peptides by microorganisms, enzymes, or chemical reaction. Taste active peptides and amino acids impart bitter, umami, or kokumi taste (Table 1-3).

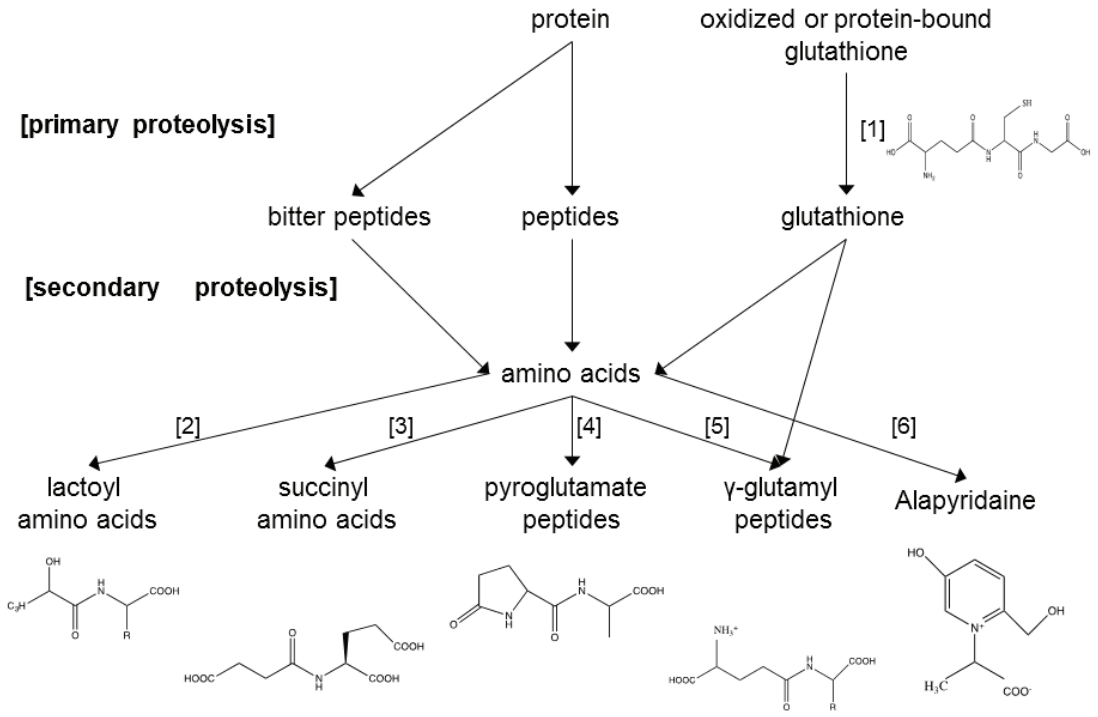

Figure 1-1. Generation of taste compounds from proteins during food fermentation. Proteolysis generates taste active peptides and amino acids; glutathione reductase generates the kokum-active glutathione. Further conversion of peptides or amino acids to taste active compounds proceeds by enzymatic reactions that are catalysed by [2] [3] [4] [5], or by thermal conversion to Amadori compounds (Alapyridaine) [6].

1.2.1 Proteolysis

Proteolysis converts protein into peptides or amino acids in different fermented foods. Proteolysis in food fermentations has been comprehensively reviewed (Gänzle et al., 2008; Hughes et al., 2002; Savijoki et al., 2006; Toldra et al., 1993a; Toldra & Flores, 1998). An overview is provided in Table 1-1.

Table 1-1 Comparison of proteolysis in different fermented food

	Primary proteolysis	**Secondary proteolysis**	**Conversion of amino acids**	**Ref.**
Cheese	LAB (surface flora)	NSLAB	*Penicillium roquefortii,* LAB	(Khalid & Marth, 1990)
Soy sauce	*Aspergillus* spp.	*Tetragenococcus halophilus*	*Aspergillus*, LAB	(Lioe et al., 2010)
Bread	Cereal enzymes (fungal or malt protease)	LAB	LAB	(Gänzle et al., 1997; Gänzle et al., 2005; Gobbetti& Gänzle, 2007; Gänzle et al., 2007; Gänzle et al., 2008; Gänzle et al., 2009)
Meat	Muscle enzymes: cathepsins, calpains, trypsin-like peptidases (surface flora)	LAB, staphylococci, surface flora	LAB, staphylococci, surface flora	(Benito et al., 2002; Benito et al., 2003; Freiding et al., 2012; Hughes et al., 2002; Sinz et al., 2013; Toldra et al., 1993a)

Table 1-2 Comparison of enzymes that generate taste active peptides or amino acids in different fermented foods.

Enzyme	Microorganisms	Food	ref
Glutaminase	*L. reuteri, Aspergillus sojae*	Sourdough, soy sauce	(Ito et al., 2013; Lioe et al., 2010; Teixeira et al., 2014)
Glutamate decarboxylase	*L. brevis, L. reuteri*	Cleese, sourdough	(Su et al., 2011; Teixeira et al., 2014)
γ-Glutamyl-transferase	*Aspergillus, Penicilliums, Bacillus. subtilis*	Cheese, soy sauce, sourdough	(Minami et al., 2003a; Minami et al., 2003b)
Glutathione reductase	*L. sanfranciscensis*	Sourdough	(Jaensch et al., 2007)
Succinyl transferase	*Aspergillus*	Soy sauce	(Frerot & Chen, 2013)
Pyroglutamyl cyclase	*L. helvetius, L. delbruechii, S.thermophilus*	Soy sauce, meat product	(Altamura, et al., 1970; Mucchetti et al., 2002)
Lactoyl-transferase (proposed, speculative or something of the like)	*Lactobacillus spp.*	Soy sauce, cheese, meat product	(Sgarbi et al., 2013)

Table 1-3 Sweet, umami and kokumi amino acids or peptides isolated from fermented food.

Name of compound	Taste threshold mmol/kg	Food source	ref
Sweet taste			
Ala	6.7	Bread, soy, meat	(Henriksen & Stahnke, 1997; Lioe et al., 2006)
Gly	25	Cheese, soy sauce	(Toelstede & Hofmann, 2008a)
Met	5	Cheese, soy sauce	(Toelstede & Hofmann, 2008a)
Pro	25	Cheese, bread, meat	(Toelstede & Hofmann, 2008a)
Ser	25	Cheese, soy sauce	(Toelstede & Hofmann, 2008a)
Thr	35	Cheese, soy sauce	(Toelstede & Hofmann, 2008a)
Ile-Glu	<5.5	Soy sauce	(Yamamoto et al., 2014)
Ile-Gln	<5.5	Soy sauce	(Yamamoto et al., 2014)
Leu-Gln	<5.5	Soy sauce	(Yamamoto et al., 2014)
Pro-Lys	<5.5	Soy sauce	(Yamamoto et al., 2014)
Thr-Phe	<5.5	Soy sauce	(Yamamoto et al., 2014)
pGlu-Gln	1.9	Soy sauce	(Kaneko et al., 2011)
pGlu-Gly	2.2	Soy sauce	(Kaneko et al., 2011)
Umami taste			
Asp	6.4	Soy sauce,cheese, bread, meat	(Khalid & Marth, 1990; Thiele et al., 2002; Toelstede & Hofmann, 2008a)
Glu	1.1, 0.3	Cheese, soy sauce, meat	(Drake et al., 2007; Henriksen & Stahnke, 1997; Toelstede & Hofmann, 2008a)
Tyr	4	Green tea	(Careri et al., 1993; Sforza et al., 2001; Sgarbi et al., 2013)
Lactoyl Glutamine	5	Cheese, soy sauce, meat	(Sgarbi et al., 2013)
Glu-Asp	7.6	Fish	(Schindler et al., 2011)
Glu-Asp-Glu	7.6	Fish	(Schindler et al., 2011)
Glu-Glu	5.4	Fish	(Schindler et al., 2011)
Glu-Gly-Ser	6.9	Fish	(Schindler et al., 2011)

Glu-Ser	8.5	Fish	(Schindler et al., 2011)
Thr-Glu	12.1	Fish	(Schindler et al., 2011)
Asp-Glu-Ser	8.6	Fish	(Schindler et al., 2011)
Glu-Gln-Glu	7.4	Fish	(Schindler et al., 2011)
Ser-Glu-Glu	5.5	Fish	(Schindler et al., 2011)
KGNEESLA	0.5	Meat	(Tamura et al., 1989; Wang et al., 1996)
γ-glutamylethylamide	24	Green tea	(Kaneko et al., 2006)
Fru-Glu	0.8	Soy sauce	(Yamamoto et al., 2014)
N-glucosyl -Glu	1.6	Soy sauce	(Beksan et al., 2003)
N-deoxyfructosyl-Glu	1.8	Soy sauce	(Beksan et al., 2003)
Kokumi taste			
γ-Glu-Glu	0.018 [a]	Cheese, bread	(Toelstede et al., 2009; Toelstede & Hofmann, 2009)
γ-Glu-Phe	2.5	Soy sauce	(Sforza et al., 2006; Toelstede et al., 2009)
γ-Glu-Gly	0.018[a]	Cheese, meat, soy sauce	(Toelstede et al., 2009; Toelstede & Hofmann, 2009)
γ-Glu-His	0.01 [a]	Cheese	(Toelstede et al., 2009; Toelstede & Hofmann, 2009)
γ-Glu-Leu	0.005 [a]	Cheese	(Sforza et al., 2006; Toelstede et al., 2009; Toelstede & Hofmann, 2009)
γ-Glu-Met	0.005 [a]	Cheese	(Toelstede et al., 2009; Toelstede & Hofmann, 2009)
γ-Glu-Gln	0.0075[a]	Cheese	(Toelstede et al., 2009; Toelstede & Hofmann, 2009)
γ-Glu-Val	0.0033 [a]	Cheese	(Toelstede et al., 2009; Toelstede & Hofmann, 2009)
γ-Glu-Tyr	2.5	Cheese, soy, meat	(Roudotalgaron et al., 1994; Toelstede & Hofmann, 2009; Toelstede et al., 2009)
γ-Glu-Cys-Gly	0.3	Sourdough	(Ueda et al., 1997)
γ-Glu-Val-Gly	0.3	Soy sauce	(Kuroda et al., 2013)

[a] The taste threshold of the compounds is measured in cheese. All the amino acids and peptides are L-type. The free amino acids and di- and tripeptides are coded in three letter.

In cheese and soy sauce, microorganisms are the major or sole contributor of protease and peptidase. α_1-Casein and β-casein are hydrolyzed by cell-wall-bound, intracellular, and extracellular proteinases from LAB. Peptides released form casein by proteinases are subsequently hydrolyzed by the intercellular peptidases of LAB. Intercellular proteinase and peptides, released during cheese ripening by autolysis, produce free amino acids and convert large peptides into small water-soluble peptides for flavor and taste development (Broadbent et al., 2002; Khalid & Marth, 1990). Extensive proteolysis of β-casein generates peptides by proteases. Imbalanced peptides that were not adequately removed by microbial peptidases might lead to a bitter taste (Fallico et al., 2005). Bitterness and debittering of amino acid and peptides will be discussed below.

In soy sauce, primary proteolysis is related to *Aspergillus*, which converts most of cereal proteins into peptides followed by LAB and yeast fermentation. Koji molds, such as *Aspergillus oryzae* and *Aspergillus sojae*, ferment soybeans into small peptides and amino acids for subsequent brine fermentation with abundant extracellular enzymes. In the following moromi fermentation, *Tetragenococcus halophilus* grows and produces lactic acid at an earlier stage to decrease the pH from 7 to less than 5. Large amounts of free glutamate, aspartate, and proline are released, which highly contribute to the taste of soy sauce (Kaneko et al., 1994). During soy sauce ripening, LAB and yeast contribute to taste and flavor generation (Kaneko et al., 2011).

Conversely, in sourdough breads and meat products, enzymes from raw materials determine the proteolysis (Gänzle et al., 2008; Ordonez et al., 1999; Toldra et al., 1993a). Meat endogenous enzymes, such as dipeptidyl peptidases (DPP) and cathepsin B, mainly contribute to proteolysis during the production of sausage or ham (Sentandreu et al., 2003).

LAB and Micrococcus are not proteolytic due to a low activity of bound proteinase (Ordonez et al., 1999). *P. pentosaceus* and *S. xylosus* are the most important microorganisms found in dry cured ham, but they do not show proteolytic activity (Molina & Toldra, 1992; Toldra & Flores, 1998). In sourdough, during secondary hydrolysis, LAB contributes significantly to the hydrolysis of peptides and amino acids transmission. The contribution of *Lactobacillus* to proteolysis is well established and the amino acid profile has been investigated (Gänzle et al., 2008).

1.2.2 Bitter peptides

Proteolysis can generate bitter peptides, depending on the amino acid composition and sequence (Kim & Li-Chan, 2006). Bitter taste is desirable when perceived in low intensity but over-accumulation of bitter peptides is considered as a taste defect. The defect of bitter taste can significantly affect economic profit of the cheese industry, making bitter peptides and debittering research a priority. Bitter peptides were determined by surface hydrophobicity that was calculated by the Q value (Arai et al., 1970; Ishibashi et al., 1987b). Peptides with hydrophobicity (Q value) >1400 cal/mole and molecular masses <6kDa might display a bitter taste (Ney, 1971). However, the Q rule excludes the effect of amino acid sequences, which influence the ability of bitter peptides to bind with taste receptors. Therefore, the Q rule shows inconsistency in determining the bitterness of peptides (Toelstede & Hofmann, 2008b). Proline is a major and distinct contributor to bitter taste of peptides (Ishibashi et al., 1988). Peptides containing proline form a ball-like shape instead of helix conformation by imino ring, therefore bind to the bitter receptor and lead to strong bitterness (Tamura et al., 1990). In cheese, most of the bitter peptides are from β-casein, which is composed of 16.7% proline. The side chains containing Gly, Ala, Val, Leu, Tyr,

and Phe always impart bitterness since those amino acids are binding determinants (Arai et al., 1970; Ishibashi et al., 1987a; Ishibashi et al., 1987b; Ishibashi et al., 1988). In di- or tripeptides, bulky hydrophobic amino acids at any position determine bitterness, whereas for larger peptides, a bitter taste is related to specific basic amino acids at the N-terminus (Kim & Li-Chan, 2006). The computer simulation revealed that presence of hydrophobic bitter amino acids, the composition of hydrophobic regions, the spatial orientation of polar group and hydrophobic regions, and the proximity between polar groups and hydrophobic regions faced within the same plane space may be major determinants for bitterness (Kiw et al., 2008). It is interesting to notice that many bitter dipeptides have ACE-inhibitory activity (Li et al., 2004; Pripp & Ardo, 2007). The majority of ACE-inhibitory di- and tripeptides have Pro in the C-terminal and branched or hydrophobic amino acids in the side chain, which are in accordance with the structural characteristics of bitter peptides.

1.2.3 Umami taste

1.2.3.1 Glutamate

Glutamate is distributed widely in fermented foods. It imparts umami taste if the concentration in the food product is above the taste threshold. Glutamate can be hydrolyzed from protein or converted by glutaminase from glutamine (Table 1-2) (Ito et al., 2013; Lioe et al., 2010). γ-Glutamyl transferase (GGT) can act as "glutaminase" if water is an acceptor; therefore glutamine can convert to glutamate. GGT from *Bacillus* and *Aspergillus* are important for glutamate conversion in soy sauce (Minami et al., 2003a; Minami et al., 2003b). During sourdough fermentation, some LAB such as *L. reuteri* has glutaminase to convert glutamine to glutamate, followed by glutamate-decarboxylase to convert glutamate to GABA (Table 1-2) (Su et al., 2011; Teixeira et al., 2014). These two enzymes are

important to the acid tolerance of LAB in sourdough fermentation and also relate to the taste of sourdough bread (Vermeulen et al., 2007).

1.2.3.2 Pyroglutamic acid and peptides

Pyroglutamic acid (pGlu) and pyroglutamyl-Pro-X peptides impart umami taste with similar activity to MSG (Figure 1-1). The presence of pyroglutamyl peptides can mask or reduce the bitterness of foods and beverages. Pyroglutamyl dipeptides are generated by cyclization reaction during heating from corresponding α-glutamyl dipeptides (α-Glu-AA) or α-glutaminyl dipeptides (α-Gln-AA) (Kasai et al., 1983). Pyroglutamyl peptides can be produced by enzymatic reaction (pGlu cyclase) from pyroglutamic acid and free amino acids (Altamura et al., 1970). *L. helveticus, L. delbrueckii subsp*. *bulgaricus*, *L. delbrueckii subsp. lactis*, and *S. thermophilus* have pGlu cyclase activity (Table 1-2) (Altamura et al., 1970; Mucchetti et al., 2002). pGlu can be released form the N-terminus of proteins and peptides by the action of a specific enzyme, such as pyrrolidone carboxyl peptidase (PCP) or l-pyro-glutamyl-peptide hydrolase (PYRase) (Mucchetti et al., 2000).

1.2.3.3 Succinyl and lactoyl amino acid derivatives

Succinyl amino acids, especially Suc-Arg and Suc-Glu from soy sauce have umami taste (Table 1-3) (Frerot & Chen, 2013). Succinyl amino acids may arise from arginine catabolic pathway (Frerot & Chen, 2013). Meanwhile, succinyl transferase from *Aspergillus oryzae* may also contribute to the generation of succinyl amino acids (Frerot & Chen, 2013). The first finding of lactoyl amino acids were patented for cheese like or umami taste (Frerot et al., 1995). Lactoyl amino acid (Figure 1-1) was produced from lactic acid and free amino acids in the presence of live or lysed *L. rhamnosus* and *L. helveticus* (Table 1-2) (Sgarbi et al., 2013). The study of lactoyl-amino acids in food is very limited

and more research is needed to understand the metabolic pathways of these amino acid derivatives (Sgarbi et al., 2013).

1.2.3.4 Amadori compounds and related products of the Maillard reaction

Amadori products are intermediate products of an advanced glycation end production as a result of glycation, which is normally associated with the Maillard reaction. Due to the long-term fermentation, Amadori products are identified in fermented foods, such as soy sauce and long ripened meat products. Amadori products, such as alapyridaine, N-glycosides, pyroglutamyl-peptides, and N-acetylglycine have an umami taste (Figure 1-1) (Ottinger & Hofmann, 2003; Shima et al., 1998; Winkel et al., 2008). Ottinger and Hofmann (2003) isolated alapyridaine, which is produced by alanine and glucose through the Maillard reaction from beef broth. Alapyridaine had a salty or umami taste enhancing activity (Ottinger & Hofmann, 2003). Malic acid glucopyranoside, which had an umami taste and little bitter taste, was isolated from Morel mushrooms (Rotzoll et al., 2005). From soy sauce, Amadori products were isolated, including Fru-Val, Fru-Met, Fru-pGlu, demonstrating astringency or bitterness, (Kaneko et al., 2011). Even though those compounds exist at subthreshold concentration, they provide the background taste of soy sauce and enhance the umami taste of glutamate (Kaneko et al., 2011).

1.2.4 Kokumi taste

1.2.4.1 Glutathione and γ-glutamyl dipeptides

Glutathione (GSH) was first identified as kokumi compound. It has enhanced continuity, mouthfulness and thickness when added to a solution containing both MSG and inosine-5'-monophosphate (Ueda et al., 1997). GSH determines bread texture due to its

effect on the gluten network (Joye et al., 2009). In fermented foods, GSH originates from oxidized glutathione by glutathione reductase from specific *Lactobacillus* (Figure 1-1) (Jaensch et al., 2007). γ-Glutamyl transferase (GGT), γ-glutamyl transpeptidase, and γ-Glu-Cys synthetase also contribute to the conversion of glutathione (Roudotalgaron et al., 1994). γ-Glutamyl transferase (GGT) catalyzes the hydrolytic cleavage of the isopeptide bond and the transfer of γ-glutamyl unit to amino acids of peptides (Toelstede & Hofmann, 2009). γ-Glutamyl dipeptides and GSH are substrates of γ-glutamyl transpeptidase and generate a large variety of γ-glutamyl dipeptides (Table 1-2) (Roudotalgaron et al., 1994).

γ-Glutamyl transferase (GGT), which contributes to the effect on glutathione, is also important to the synthesis of γ-glutamyl peptides and produces glutamate (Table 1-2). A series of kokumi peptides are isolated from cheese. The generation of kokumi peptides is attributed to γ-glutamyl transferase and/or γ-Glu-Cys synthetase activity of surface molds (Table 1-3) (Roudotalgaron et al., 1994). Proteolysis of casein only liberates α-bond proteins or peptides (Roudotalgaron et al., 1994). In GGT reaction, acidic and basic amino acids are poor acceptors, whereas neutral amino acids are preferred (Toelstede & Hofmann, 2009). GGT is found in *Penicillium* spp. in cheese, and in *Aspergillus* or *Bacillus* from soy sauce. However, recent studies indicated endogenous enzymes from milk and some *Lactobacillus* also show GGT activity, but the contribution of GGT to the taste of fermented foods remains unclear (Table 1-2) (Arai et al., 1973; Sgarbi et al., 2013; Toelstede & Hofmann, 2009). It is also possible that another pathway or unknown enzyme apart from GGT is related to the generation of γ-glutamyl dipeptides (Toelstede & Hofmann, 2009). γ-Glutamyl peptides have a higher solubility in water and also resist hydrolysis by peptidases (Suzuki & Kumagai, 2004b).

1.3 Contribution of taste active amino acids and peptides to food

1.3.1 Soy sauce

Soy sauce, with typical umami and salty taste, is consumed widely as a condiment. The typical umami taste of soy sauce is attributed to the free amino acids hydrolysed from soy protein and wheat gluten, especially Glu, Ala, and Asp (Table 1-3) (Kaneko et al., 2011; Lioe et al., 2004; Lioe et al., 2006). Although some small peptides including pGlu-Asp, pGlu-Val, and lac-Glu exhibit umami taste in soy sauce, due to their low concentration in soy sauce, their direct contribution is negligible. However, those compounds provide the taste background and enhance other tastes (Noguchi et al., 1975; Oka & Nagata, 1974a; Oka & Nagata, 1974b; Frerot & Chen, 2013) (Table 1-3). Omission and reconstitution tests indicated that pyroglutamyl peptides and Amadori compounds contributed to the umami taste at sub-threshold concentrations (Frerot & Chen, 2013). Shiga et al (2014) firstly identified Fru-Glu from 25 different soy sauces. Fru-Glu increased the intensity of the umami taste at the subthreshold level due to a strong synergistic effect between Fru-Glu and glutamate (Shiga et al., 2014).

1.3.2 Cheese

Cheese has a very complex and characteristic flavor, taste, and texture. Compounds that contribute to cheese taste and flavor are produced as a consequence of biochemical changes during cheese ripening. The free amino acids, small peptides, and amino acid derivatives, such as lactoyl amino acids, and pyroglutamyl amino acids, are found in cheese (Table 1-3). All of those compounds impart the umami taste and enhance other tastes (Sforza et al., 2009). The kokumi taste, together with the umami taste, contributes to the characteristic taste of cheese (Table 1-3). Unbalanced bitterness is unpleasant to the consumer and thus is a serious economic concern for the cheese industry (Table 1-4).

α-Glutamyl di- and tripeptide, especially Asp-, Thr- and Ser-containing peptides, have umami taste (Table 1-3). The taste of α-Glu-X is highly determined by hydrophobicity of the second amino acid. For example, Glu-Asp, Glu-Thr, Glu-Ser, Glu-Glu and Glu-Gly-Ser have umami taste; Glu-Gly, Glu-Ala, Glu-Pro and Glu-Val have flat or no taste; Glu-Ile, Glu-Leu, Glu-Tyr and Glu-Phe possess bitter taste (Table 1-3 and Table 1-4) (Arai et al., 1973). Other dipeptides, including Arg-Pro, Asp-Asp, Arg-Asp, pGlu-Gln, pGlu-Gly, Asp-Glu, Glu-Glu, also have umami taste in cheese. Glu-enriched hydrophilic oligopeptides, especially with Glu- residue in N-terminus or C-terminal, possess umami taste (Kim et al., 2015). The taste of α-Glu-X is not related to γ-Glu-X. On the contrary, polarity, acidity, and hydrophobicity are essential criteria for the umami taste. α-Glutamyl dipeptides are formed by proteolysis of casein, whereas γ-glutamyl dipeptides are generated by γ-glutamyl transpeptidase or γ-glutamyl transferase from amino acids released upon protein hydrolysis (Toelstede et al., 2009; Toelstede & Hofmann, 2009).

Table 1-4 Bitter amino acids and peptides in fermented foods

Name of compound	Taste threshold mmol/kg	Food esource	Ref
Arg	75	Cheese	(Toelstede & Hofmann, 2008a)
His	1.2	Cheese, sausage	(Henriksen & Stahnke, 1997; Toelstede & Hofmann, 2008a)
Ile	10	Cheese, sausage	(Henriksen & Stahnke, 1997; Hughes et al., 2002; Toelstede & Hofmann, 2008a)
Leu	11	Cheese, sausage	(Henriksen & Stahnke, 1997; Hughes et al., 2002; Toelstede & Hofmann, 2008a)
Lys	3.4	Soy sauce, meat	(Kato, Rhue, & Nishimura, 1989; Toldra & Aristoy, 1993b)
Phe	45	Cheese, soy sausage	(Henriksen & Stahnke, 1997; Hughes et al., 2002; Sgarbi et al., 2013; Toelstede & Hofmann, 2008a)
Try	4	Cheese	(Toelstede & Hofmann, 2008a)
Tyr	4	Cheese	(Toelstede & Hofmann, 2008a)
Val	3.4	Cheese, sausage	(Henriksen & Stahnke, 1997; Kato, Rhue, & Nishimura., 1989; Toelstede & Hofmann, 2008a)
Fru-Met	1.6	Soy sauce	(Kaneko et al., 2011)
Fru-Val	1.8	Soy sauce	(Kaneko et al., 2011)
Fru-pGlu	2.6	Soy sauce	(Kaneko et al., 2011)
Met-Ile	0.42	Cheese	(Toelstede & Hofmann, 2008b)
Glu-Ala	10	Cheese	(Roudotalgaron et al., 1994a; Toelstede & Hofmann, 2009; Toelstede et al., 2009)
Glu-Gly	2.5	Cheese	(Toelstede & Hofmann, 2009; Toelstede et al., 2009)
Glu-Thr	2.5	Cheese	(Roudotalgaron et al., 1994a; Toelstede & Hofmann, 2009; Toelstede et al., 2009)
Glu-Val	5	Cheese	(Roudotalgaron et al., 1994a; Toelstede & Hofmann, 2009; Toelstede et al., 2009)

Name of compound	Taste threshold mmol/kg	Food esource	Ref
Glu-Trp	5	Cheese	(Roudotalgaron et al., 1994a; Toelstede & Hofmann, 2009; Toelstede et al., 2009)
Glu-Tyr	5	Cheese	(Roudotalgaron et al., 1994a; Toelstede & Hofmann, 2009; Toelstede et al., 2009)
LPQE	0.6 [a]	Cheese	(Toelstede & Hofmann, 2008a)
DIKQM	0.6 [a]	Cheese	(Toelstede & Hofmann, 2008a)
EIVPN	0.43 [a]	Cheese	(Toelstede & Hofmann, 2008b)
VRGPFP	0.42 [a]	Cheese	(Toelstede & Hofmann, 2008b)
GPVRGPFP	1.18 [a]	Cheese	(Toelstede & Hofmann, 2008b)
YPFPGPIHN	0.1 [a]	Cheese	(Toelstede & Hofmann, 2008b)
YPFPGPIPN	0.33 [a]	Cheese	(Toelstede & Hofmann, 2008b)
VYPFPGPIPN	0.17 [a]	Cheese	(Toelstede & Hofmann, 2008b)
YPFPGPIHNS	0.05 [a]	Cheese	(Toelstede & Hofmann, 2008b)
YPFPGPIPNS	0.33 [a]	Cheese	(Toelstede & Hofmann, 2008b)
LVYPFPGPIHN	0.08 [a]	Cheese	(Toelstede & Hofmann, 2008b)
SLVYPFPGPIHNS	0.06 [a]	Cheese	(Toelstede & Hofmann, 2008b)
YQQPVLGPVRGPFPIIV	0.18 [a]	Cheese	(Toelstede & Hofmann, 2008b)

[a] The taste threshold of the compounds is measured in cheese. All the amino acids and peptides are L type. The free amino acids, di- and tripeptides are written in three letter code while the single letter code is used for peptides larger than tripeptides.

γ-Glutamyl peptides with kokumi taste, such as γ-Glu-Phe, γ-Glu-Tyr and γ-Glu-Leu, were first isolated from Comte cheese (Roudotalgaron et al., 1994). In ripened Gouda cheese, γ-Glu-Glu and α-Glu-Glu were the most abundant dipeptides (Toelstede et al., 2009). The concentration of the γ-glutamyl dipeptides was at least 10 times higher in 44 week ripened cheese compared to 4 week aged cheese, whereas the concentration of α-glutamyl dipeptides was only 1-8 times higher (Toelstede et al., 2009). The concentration of γ-glutamyl in cheese is about 100 to 1000 times lower than the determined taste threshold of the kokumi taste. However, the omission and reconstitution test indicate that the γ-glutamyl peptides at the subthreshold level can impart kokum sensation and enhance other tastes, which might be due to taste interactions in the food matrix and the effect of pH (Roudotalgaron et al., 1994; Toelstede & Hofmann, 2008b; Toelstede et al., 2009; Toelstede & Hofmann, 2009). Therefore γ-glutamyl peptides still play an important role in the taste profile of cheese as taste enhancer and contribute to the complex taste of cheese.

Bitterness in cheese is an economic concern. The occurrence of bitterness is attributed to unbalanced levels of proteolysis and peptidolysis (Table 1-4). Peptides are major contributors to bitter taste as proven by an omission test (Engel et al., 2001a; Engel et al., 2001b). However, the chemical environment, such as salt levels and pH values, also affect the bitterness perception (Engel et al., 2001b; Engel et al., 2001c). Peptides are not systematically responsible for bitterness, and other taste compounds might disturb the perception of bitter stimuli (Engel et al., 2001b; Engel et al., 2001c). The taste of individual peptides is probably weak or even undetectable, but their exact role in eliciting an intense bitter taste remains unclear. However, the omission of peptides and amino acids in a model led to a significant decrease in its bitter taste (Broadbent et al., 2002).

1.3.3 Fermented meat

Taste of high quality, fully matured hams results from enzymatic reactions, including proteolysis and lipolysis, and chemical processes, including lipid auto-oxidation, and Strecker and Maillard reactions throughout ripening. Free amino acids play a crucial role in producing taste and flavor of ham and sausage, but the contribution of small peptides should not be neglected (Herranz, Fernandez, de la Hoz, & Ordonez, 2006; Jurado, Garcia, Timon, & Carrapiso, 2007; Sentandreu et al., 2003; Sforza et al., 2001; Sforza et al., 2006). Although the effect of microorganisms in the proteolysis of meat protein is very limited, LAB and surface molds determine the typical flavor and taste, due to transformation and decarboxylase activity (Table 1-1) (Herranz et al., 2006; Sinz et al., 2013).

Free amino acids contribute to taste directly (Sentandreu et al., 2003). Glu and Asp are the most abundant free amino acids in fermented meat and they are present at levels above the taste threshold, thus play an important role in the taste properties of the fermented meat (Table 1-3) (Jurado et al., 2007). The concentration of predominant amino acids, such as Pro, Ala, Val, Ile, Leu, and Phe, increase above their taste threshold during sausage ripening. Therefore, free amino acids contribute significantly to taste (Kato et al., 1989). Lys, Tyr, Asp, Ala, and Glu were the most abundant free amino acids in the ripening of Iberian ham and have a strong influence on ripened taste of dry cured products (Careri et al., 1993; Hughes et al., 2002; Toldra & Aristoy, 1993b).

Peptides in meat also contribute to taste (Reina et al., 2014). Dipeptides, such as Ile-Val, Leu-Gly, Ile-Asp and Pro-Leu, were isolated from ripened ham with a bitter taste (Table 1-4) (Sentandreu et al., 2003). The bitter peptides in meat result from a low activity of aminopeptidase, which cleaves peptides into free amino acids with less bitterness (Reina

et al., 2014). High endopeptidase activity leads to a high content of Met, Asn and Ile, which impart bitterness in aged hams (Sforza et al., 2001; Sforza et al., 2006). The general concentration of bitter peptides and amino acids in muscle protein are lower than that in cheese casein; therefore, bitterness is not a major concern for meat products compared to cheese (Henriksen & Stahnke, 1997). Di- and tri-peptides with the umami or kokumi taste are found in meat products as well, especially γ-Glu-Phe in ham, which undergoes a sharp increase over extended aging times (Suzuki et al., 2002).

Peptides, amino acids and other compounds contribute to taste in a complex manner, exceeding taste properties of pure compounds due to taste interactions (Henriksen & Stahnke, 1997). Small peptides and free amino acids contribute to taste impressions as a mixture and individually. Taste-taste interaction is very important for sensory experience in the food matrix and more research is needed.

1.3.4 Bread

Investigations on the effect of amino acids or peptides on bread taste are very limited. Due to the low concentration of glutamate in straight dough, the effect of glutamate on bread taste was previously neglected (Fujisawa & Yoshino, 1995; Johnson & Eldash, 1969). Type II sourdough, which are typically fermented for 1-7 days at relatively higher temperature, are fermented for use as baking improvers. Hyrolysis of proteins during sourdough fermentation accumulates amino acids and peptides. In sourdough, the level of glutamate ranges from 27 to 120 mmol/kg, depending on the strain specificity (Vermeulen et al., 2007a). A trained panel found sourdough with 47 mmol/kg glutamate significantly influenced bread taste (Vermeulen et al., 2007a). However, the specific contribution of glutamate on bread taste is still unclear. Type I sourdough are typically fermented by

Lactobacillus sanfranciscensis. The type I sourdough are used as dough levening and fermented no more than 30°C and less for 48 h. Glutathione, as mentioned before, has a typical kokumi sensation (Ueda et al., 1997). Glutathione in bread is generated by glutathione reductase from *Lactobacillus sanfranciscensis* during Type I sourdough fermentation (Table 1-2). Glutathione integrates into the glutenin macropolymer through disulfide bonds where it acts as a chain terminator for polymer formation and determines dough rheology, gas retention, and bread volume and texture (Grosch & Wieser., 1999; Weegels et al.,1996). However, the effect of glutathione on bread taste is unclear.

1.4 Interaction between salt and taste active amino acid/peptides

NaCl plays an important role in soy sauce, cheese, meat products, and bread. It not only provides the salty taste, but it can also mask metallic and bitter tastes and enhance the umami taste. Amino acids and peptides can enhance salty taste and thus can be used to reduce salt levels. A positive correlation between the amount of glutamate and aspartate with salty taste was investigated in dry cured meat (Careri et al., 1993). An omission test on Gouda cheese indicated that arginine at subthreshold concentration significantly enhanced salty taste (Toelstede & Hofmann, 2008a; Toelstede et al., 2009). In soy sauce, Phe and Tyr at subthreshold concentrations significantly increased the salty and umami taste of the MSG/NaCl mix (Lioe et al., 2004; Lioe, Apriyantono, Takara, Wada, & Yasuda, 2005). Perception of salty and umami tastes is based on distinct receptors. However, due to the taste-taste interaction, when both umami and salty stimuli are mixed, they can generate a more intense umami or salty perception (Lioe et al., 2005). Phe at subthreshold levels may interact with two taste receptors and only significantly affect the MSG and NaCl

mixture. This possible effect provides an insight that a subthreshold taste compound can affect the intensity of other taste attributes (Lioe et al., 2005).

1.5 Debittering of food protein hydrolysates

Bitterness limits the acceptance and marketing of food, especially for cheese. Many attempts have been made to decrease the bitterness in food. Physical methods have been performed including the adsorptioin of bitter peptides on activated carbon, chromatographic removal using different matrices, and selective extraction with alcohols. The drawback of these methods is the loss of some amino acid residues. The use of masking agents such as cyclodextrin and polyphosphate is another method. The plastein reaction was used to crosslink the bitter peptides by transglutaminase. However, the application is limited by the low solubility of product (FitzGerald & O'Cuinn, 2006).

Since bitter peptides are produced by excessive or unbalanced proteolysis, effective control over this problem requires enzymes that hydrolyze bitter peptides into smaller, non-bitter peptides or free amino acids (Broadbent & Steele, 2007). The most effective strategy to control bitterness in cheese is to develop a starter system that combines a low propensity for the production of bitter peptides with a high level of debittering peptidase activity (Broadbent et al., 2002). Therefore the application of peptidases, especially exopeptidases including amino- and carboxy-peptidases, to debitter peptides is well studied. The bitterness of casein hydrolysates was significantly reduced in the presence of amino peptidases and post-proline dipeptidyl aminopeptidases (PPDA), which release amino acyl proline residues from N-terminus (Bouchier et al., 2001). Cell free extracts of different *L. lactis ssp. cremoris* were used for debittering peptides due to the activity of PepXP, which can cleave the X-Pro-Y peptide bond and liberate X-Pro from bitter peptides (Shimamura

et al., 2009). Moreover, aminopeptidases derived from *Aeromonas caviae* T-64 also showed a debittering effect. In cheese, *L. helveticus* CNRZ32 had a significant debittering effect that was attributed to post-prolyl endopeptidases, such as $PepO_2$, $PepO_3$, and PepF (Sridhar et al., 2005). Pro-containing bitter peptides were converted into smaller peptides that can be efficiently degraded to free amino acids and non-bitter X-Pro dipeptides by general aminopeptidase PepN (Broadbent & Steele, 2007).

Another new strategy is to convert bitter amino acids into γ-glutamyl derivatives through GGT. This strategy has two benefits. First of all, bitterness was decreased because most γ-glutamyl dipeptides have the kokumi or umami taste (Suzuki et al., 2002). For example, the bitterness of Phe, Val, Leu and His was reduced significantly and preference was increased in the form of γ-glutamyl derivatives (Suzuki et al., 2002; Suzuki & Kumagai, 2004b; Suzuki et al., 2004a). Secondly, γ-glutamyl peptides with the umami or kokumi taste suppress the bitter taste due to umami-bitter interaction (Son et al., 2015). High levels of Glu, Ser, Gly, His, Ala, Met, and Lys can mask the bitterness of phenylalanine (Henriksen & Stahnke, 1997). Five representative umami peptides suppressed sialicin-induced intracellular calcium influx in a noncompetitive manner. Glu-Glu at 1 mM was the most effective inhibitor of salicin induced intracellular Ca^{2+} response and hence inhibited the bitter taste sensation (Kim et al., 2015). MSG with adenosine monophosphate or sodium salt of 5'-ribonucleotides inhibits bitter tastes (Kemp & Beauchamp, 1994). Umami active oligopeptides containing glutamyl peptides, including Glu-Asp, Glu-Glu, Glu-Ser, and Glu-Glu-Glu, suppress the bitter taste of the protein hydrolysates (Tokita & Boughter, 2012).

1.6 Perspectives of future research

The generation and contribution of taste active amino acids and peptides during food fermentation are well elucidated, but some pathways are still unknown. Taste-taste interactions are important for food product development. Subthreshold compounds also play a role in sensory characteristics of food products. This knowledge indicated that food fermentation is a very complex system, which generates specific taste and flavor of amino acids and peptides profile. Also, those taste active compounds can interact within food matrix and produce the typical taste of food products. Food quality is highly determined by specific microorganisms and enzymes during food fermentation. With the combination of the knowledge of interaction between taste active compounds and of the generation pathways, it is possible to develop fermentation strategies to decrease the fermentation time and develop tastier, less bitter, and lower salt food products.

1.7 Aim of the research

This thesis aimed to test hypotheses that free amino acids and γ-glutamyl dipeptides may accumulate during sourdough fermentation and influence the taste of bread. Also, according to previous research work, the ACE-inhibitory tripeptides are accumulated during sourdough fermentation, but the stability of those tripeptides during thermal treatment is unclear. In order to investigate these hypotheses, the following objectives were addressed:

- To quantify the level of free amino acids, especially glutamate in sourdough and final bread (Chapter 2).

- To understand the effect of glutamate on the taste of bread by comparing the taste of bread made by isogenic mutant *L. reuteri* 100-23 and *L. reuteri* 100-23 wild type (Chapter 2).
- To study if sourdough fermented by different *L. reuteri* can generate different types of kokumi peptides and influence the taste (Chapter 3).
- To understand the effect of thermal treatment on the stability of ACE-inhibitory tripeptides by analysing the level of ACE-inhibitory tripeptide in steamed bread, soda cracker and baked bread using LC-MS/MS in MRM mode (Chapter 4).

2 Effect of glutamate-accumulation during sourdough fermentation with *L. reuteri* on the taste of bread and sodium-reduced bread

2.1 Introduction

The reduction of dietary salt intake has consistently been shown to reduce cardiovascular disease risk (Cook et al., 2007; He & MacGregor, 2011). Therefore, the World Health Organization recommended a maximum daily intake of NaCl for adults of 5 g/day. However, currently, the average daily salt intake in industrialized nations ranges from 9 to 12 g/day (World Health Organization. 2007). Cereal products, including bread, are major sodium contributors (Public Health England, 2003). In Germany, bread accounts for 24% of the daily sodium intake (Max Rubner-Institut, 2008). Therefore, bread is a suitable target to reduce the dietary intake of sodium.

NaCl tastes salty, masks metallic and bitter tastes, and enhances flavor. NaCl also influences the texture of wheat bread because it strengthens the gluten network at the dough stage (Miller & Hoseney, 2008; Salovaara, 1982a). Because NaCl plays multiple roles for sensory and technological properties of bread, it is challenging to reduce the sodium content without compromising taste, volume, and texture. Sodium reduction by 25% in bread decreased liking (Girgis et al., 2003). Replacement of 20% NaCl with KCl and MgCl allowed the production of bread with acceptable taste (Salovaara, 1982b). An inhomogeneous spatial distribution of sodium in bread also allowed sodium content reduction without reducing salty taste intensity (Noort et al., 2010).

The use of taste enhancers including yeast extract, nucleotides and glutamate, enhanced the salty taste of soup. Glutamate in soup improved the sweetness and saltiness (Yeomans et al., 2008); glutamate addition also allowed salt reduction by 30-40% without influencing palatability (Yamaguchi & Takahashi, 1984). However, all these strategies were applied rather empirically and no data are available for bread.

Type II sourdoughs are fermented for use as baking improvers. Hydrolysis of proteins during sourdough fermentation accumulates amino acids and peptides (Stromeck et al., 2011; Thiele et al., 2002). Glutamine is converted to glutamate or γ-aminobutyrate (GABA) during fermentation (Stromeck et al., 2011; Vermeulen et al., 2007). *L. reuteri* TMW1.106 accumulates glutamate while the glutamate decarboxylase positive *L. reuteri* LTH5448 and 100-23 further decarboxylate glutamate to GABA (Stromeck et al., 2011). However, the effect of glutamate accumulation during sourdough fermentation on the taste of bread has not been determined.

It was therefore the aim of this study to evaluate the effect of glutamate accumulation during sourdough fermentation on the taste of bread. Sourdough was fermented with strains of *L. reuteri* accumulating glutamate or GABA as alternative end products of glutamine metabolism. Additionally, *L. reuteri* 100-23 and the isogenic mutant *L. reuteri* 100-23Δ*gadB,* which lacks glutamate decarboxylase activity and accumulates glutamate in sourdough fermentations were employed (Su et al., 2011). Bread was produced with a NaCl content of 1 – 2 % and sensory evaluation was performed with trained panels and untrained consumer panels. The technological properties of the bread were analyzed and compared with the reference bread.

2.2 Material and methods

2.2.1 Strains and growth conditions

L. reuteri TMW1.106, LTH5448, 100-23, and 100-23ΔgadB were routinely cultivated in mMRS media at 37 °C. Strains were subcultured twice with 1% inoculum in food grade wort medium containing 5% (w/v) CBW Munich malt extract (BRIESS malt&ingredients Co., Chilton, USA) to inoculate sourdough fermentations.

2.2.2 Preparation of sourdough

Rye malt was kindly provided by Laihian Mallas (Laihia, Finland). Whole wheat flour and vital wheat gluten were purchased in a local supermarket. Rye malt sourdoughs were prepared and fermented for 96 h as previously described with minor modifications (Stromeck et al., 2011). To obtain food-grade sourdough, the starter cultures were prepared in wort medium and sourdoughs were inoculated with overnight cultures in wort medium. Fermentations were carried out in a food preparation laboratory. Cell counts and the pH of sourdough were determined every 24 h. After 96 h of fermentation, the sourdough was freeze dried and stored at room temperature for subsequent use in baking experiments and for analysis of amino acid concentrations. The fresh sourdough was used directly for DNA isolation and metabolite analysis by HPLC. The sourdough was fermented in duplicatae independent fermentation and all the analysis were in triplicate.

2.2.3 Preparation of sourdough and reference breads

Sourdough bread was prepared by replacing 3 or 6% of the whole wheat flour with dried rye malt gluten sourdough fermented with *L. reuteri* TMW1.106, LTH5448, 100-23 or 100-23Δ*gadB*. Sourdough bread contained 0.5, 1, or 2% NaCl (flour basis) for triangle

test. Reference bread was prepared with a straight dough process with whole wheat flour, 2% sugar, 2% yeast, 60% water, and addition of 1%, 1.5% or 2% NaCl (percentages indicate % flour basis, i.e. g of ingredient per 100 g of flour) for ranking test. Ingredients were mixed in a spiral kneader (Kitchen Aid K45SS, Hobart Co. Troy, OH) for 3 min. The dough was shaped after a dough rest of 30 min and proofed for 90 min at 30°C and 85% humidity in a proofer (Res-Cor, Crescent Metal Products Inc, Cleveland, OH). Bread was baked in a multi-deck oven (Bakers Pride, Lachine, QC, Canada) set at a temperature of 210°C for 50 min. After baking, bread was cooled for 2 h at room temperature, packed in polyethylene bags, sealed, and stored frozen at -18°C. Prior to sensory evaluation, bread was thawed at room temperature for 24 h. All the baking trials were carried out triplicate.

Bread for the trained panel evaluation was baked in at a pilot scale facility using the dough formula shown in Table 2-1. Reference breads were prepared with a straight dough process (control), with inclusion of rye malt and gluten, or with inclusion of a commercial sourdough (Fermdor Germ Sourdough, Bakels Nutribake AG, Switzerland) (Table 2-1). Bread prepared for training of the panel and the evaluation of the taste threshold of glutamate in bread contained 0%, 0.0075%, 0.015%, 0.03%, 0.06%, 0.12% or 0.18% monosodium glutamate (flour basis) (Table 2-1). Scalding was prepared by mixing coarse whole wheat flour with four times the weight of water and placing the mixture in a pan with a lid in an oven (Condo, Miwe, USA) at 200 °C for 2 h with stirring after 60 and 90 min. Ingredients for bread baking, including the cold scalding (Table 2-1) were mixed in a kneader (Diosna Labkneader SP4, Diosna, USA) for 10 min at 20 Hz and 4 min at 45 Hz. The dough was proofed for 2 h at room temperature, divided into 1400 g pieces that were shaped into loaves, and finally proofed for 45 min at 35 °C and 75% relative humidity.

Bread was baked at 240 °C for 15 min with 360 mL steam, 230 °C for 20 min and finally for 15 min with open vent (Condo, Miwe, USA). Prior to evaluation by the trained panel, the bread was stored overnight at room temperature. All the baking trials were carried out triplicate.

Table 2-1 Recipes for preparation of bread for trained panel.

Addition per batch (kg)	**Control**	**Control + MSG**	**Rye-malt gluten**	**Commercial sourdough**	***L. reuteri* sourdoughs[a)]**
Fine whole wheat flour	1.627	2.968	1.52	1.484	1.484
Scalding from coarse whole wheat flour[a)]	0.569	1.138	0.569	0.569	0.569
Water	0.936	1.869	0.936	0.936	0.936
Yeast	0.036	0.075	0.036	0.036	0.036
Salt (2.0% or 1.0%)	0.036 or 0.018	0.071	0.036 or 0.018	0.036 or 0.018	0.036 or 0.018
Monosodium glutamate		0 – 0.006[c)]			
L. reuteri sourdough[b)]					0.143
Commercial sourdough				0.143	
Rye malt flour			0.054		
Wheat gluten			0.054		

[a)] Scalding was prepared with 0.4065 kg water and 0.1625 kg coarse whole wheat flour. The recipe was doubled for preparation of bread with MSG.

[b)] Rye-malt gluten sourdough fermented for 96 h with *L. reuteri* TMW1.106, LTH5448, 100-23 or 100-23ΔgadB

[c)] addition of 0, 0.27, 0.53, 1.06, 2.12, 3.18, or 6.36 g monosodium glutamate to the bread dough corresponding to a MSG level of 0.0075, 0.015, 0.03, 0.06, 0.12 or 0.18% (weight / flour weight) monosodium glutamate, respectively.

2.2.4 DNA isolation and high-resolution melt curve quantitative PCR (HRM-qPCR)

The identity of the sourdough fermentation microbiota with the inoculum was verified by high resolution melt quantitative PCR as described previously (Lin & Gänzle, 2014). In brief, DNA was isolated from 1 mL 96 h sourdough or overnight cultures of *L. reuteri* grown in mMRS using a DNeasy Blood and Tissue kit according to the instructions of the manufacturer (Qiagen, Mississauga, ON, Canada). HRM-qPCR (Rotor-GeneQ, Qiagen, Mississauga, ON, Canada) was used to amplify the 16S rRNA genes of *L. reuteri*. PCR reactions were performed in a volume of 10 µL containing 1 µL of template DNA, 5 µL of Master mix (Type-it HRM PCR kit, Qiagen, Mississauga, Canada), 3.86 µL of autoclaved MilliQ water, 0.07 µL of primers (IDT, Coralville, IA, USA) 16S-F (AGA6TTTGATYMTGGCTC) and 16S-R (CAKAAAGGAGGTGATCC). The PCR conditions were: denaturation 5 min at 95°C, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 30 sec, and extension at 72°C for 10 sec. At the final HRM stage, the temperature increased from 70°C to 90°C at 0.1°C/step with 2 sec holding time at each step. The DNA from all samples was extracted duplicately and PCR were carred out at once.

2.2.5 Quantification of metabolites by HPLC

Organic acids, ethanol, and monosaccharides in sourdough were quantified in triplicate by high-performance liquid chromatography (1200 series, Agilent Technologies, USA) equipped with an Aminex HPX 87H column as described (Bio-Rad) by Dlusskaya et al (2008). Glucose, maltose, lactate, acetate, mannitol and ethanol (all from Sigma) were used as external standards. Amino acids in sourdough and in bread were quantified as

described by Stromeck et al. (2011). In brief, lyophilized samples were mixed with water at 1:20 (w/v) ratio and extracted for 1 h at room temperature. Solids were removed by centrifugation at 10,000 *rcf* for 10 min. Amino acids were quantified after derivatization with o-phthaldialdehyde as described by Sedgewick et al. (Sedgwick et al., 1991). β-Aminobutyric acid was used as an internal standard. Amino acid concentrations in sourdough were analysed with samples from triplicate independent fermentations and each dough was analyzed in duplicate. Amino acid concentrations in bread were analysed with samples from two independent batches of breads that were analyzed duplicate.

2.2.6 Sensory evaluation by consumer panels

The sensory studies were reviewed for their adherence to ethical guidelines and approved by the Research Ethics Board at the University of Alberta. Bread crumb was cut into 1cm^3 pieces avoiding inclusion of bread crust. Four pieces of bread were placed separately in covered plastic dishes labeled with 3-digit random numbers and randomly assigned to each panelist. Panelists (40) were recruited from staff and students at the University of Alberta. Filtered water and 3 pieces of apple were provided to cleanse the palate between samples. Conventional triangle tests (Lawless & Heymann, 1998) were conducted to compare bread produced with rye-malt gluten sourdough fermented with *L. reuteri* 100-23 and *L. reuteri* 100-23*ΔgadB; L. reuteri* LTH5448 and *L. reuteri* TMW1.106; or *L. reuteri* LTH5448 and *L. reuteri* 100-23 for each level of sourdough and salt addition. The numbers of correct responses required for significance were determined at $p<0.05$ (Meilgaard et al., 1999). In order to understand the effect of salt reduction of sourdough, *L. reuteri* 100-23 or 100-23Δ*gadB* sourdough bread inclusion of 6% sourdough and 1%

NaCl, reference bread at 1%, 1.5% or 2% NaCl were ranked based on the intensity of saltiness (Takahashi et al., 2002). Friedman test were used to analyse the result.

2.2.7 Sensory evaluation by trained panels

The panel consisted of 9 subjects (5 woman and 4 men, aged 25-35 years) who routinely participate in bread sensory evaluation panels. In the first of two training sessions, the panelists were accustomed to monosodium glutamate (MSG) solution in water at concentrations of 0, 0.003, 0.01, 0.02, 0.03, 0.035, 0.04, 0.05, 0.06 and 0.065% (w/v). The lowest MSG concentration identified as different from water was determined to be the taste threshold of MSG in water. In the second session, the panelists were accustomed to MSG in bread to determine the threshold in the same way.

Descriptive sensory analyses (sweet, salty, sour, bitter and umami) were conducted in 4 different sessions in a meeting room. Bread contained 1% or 2% salt and the evaluation was done with bread slices both including and excluding the crust, according to the method of Arthur (Meilgaard et al., 1999). In brief, the panel evaluated each attribute on a 10-point category scale (0=weakest, 9=strongest). A 10-point scale was chosen as it can avoid the error of central tendency. The panel leader led a general discussion of the panel to arrive at a consensus profile for each sample.

2.2.8 Analysis of bread quality

To characterize the impact of the sourdough on the volume and texture of bread, the volume, pH, titratable acidity, dry matter content, and the water activity were measured. Measurements were performed with bread obtained in two independent baking trials with triplicate independent measurements of each. The specific volume of bread was measured with BreadVolScan (Backaldrin, Asten, Austria). The pH of bread was measured by a pH

electrode (Titrino plus 848, Metrohm, USA). Total titratable acidity (TTA) was expressed as the amount in mL of 0.1M NaOH to obtain pH 8.5. Dough stickiness and bread texture were measured with the TA-XTplus Texture Analyzer (Stable Micro Systems, Great Britain) with 9 replications and 6 replications, respectively, for two independent samples.

2.2.9 Statistical analyses

Statistical analysis of metabolite concentrations in sourdoughs was performed by analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons. Statistical analysis of technical properties of bread was performed by analysis of variance (ANOVA) with the procedure PROC GLM using the Statistical Analysis System V.9.2 (SAS Institute Inc., Cary, NC). Values were considered significantly different at a 5% error level ($P < 0.05$).

2.3 Results

2.3.1 Characterization of sourdoughs

The identity of the respective fermentation microbiota with the inoculum was verified by observation of a uniform colony morphology and HRM-qPCR. All colonies obtained from sourdoughs exhibited a uniform colony morphology matching the inoculum (data not shown). HRM-qPCR with template DNA from sourdoughs generated amplicons with a size and melting temperature matching the amplicons from *L. reuteri* (Table 2-2). To monitor the sourdough fermentation and to determine the concentration of taste-active microbial metabolites, cell counts, the pH, and metabolite concentrations were quantified (Table 2-2). Cell counts and pH were comparable with results previously obtained with the same strains (Stromeck et al, 2011). After 96 h of fermentation, sourdoughs contained high

concentrations of ethanol and lactate but low amounts of acetate and mannitol. Metabolite concentrations and the pH of sourdoughs fermented with different strains of *L. reuteri* were not significantly different, excluding that strain specific differences in carbohydrate metabolism influenced the sensory properties of bread.

Table 2-2. Fermentation parameters of sourdoughs fermented for 96 h.

	pH	Cell count ($x10^7$)	T_m (°C)[b)]	Metabolites (mmol / kg)[a)]			
				Ethanol	Lactate	Acetate	Mannitol
TMW1.106	3.54±0.02	1.6±0.2	85.65	107±4	95±7	5±1	4±1
LTH5448	3.65±0.02	0.6±0.3	85.65	105±15	89±10	6±1	5±1
100-23	3.52±0.05	2.6±2.1	85.65	110±12	94±12	5±0	5±1
100-23Δ*gadB*	3.54±0.05	7.0±1.7	85.65	118±25	96±21	7±2	5±1

[a)] The concentrations of the same metabolites in sourdoughs fermented with different strains of *L. reuteri* were not significantly different ($p>0.05$).

[b)] Melting temperature of amplicons obtained with primers targeting eubacterial 16S rRNA genes and DNA isolated from the sourdough after 96 h of fermentation. The melting temperature of the amplicons obtained with all four *L. reuteri* strains was 85.65°C.

2.3.2 Amino acid concentrations in sourdoughs

The conversion of glutamine to glutamate or GABA in lactobacilli is strain specific; therefore 4 strains were selected for their ability to convert glutamine to GABA or glutamate. Amino acid concentrations in the four sourdoughs were generally comparable; however, the concentration of glutamate and GABA was strain specific. In agreement with previous reports, *L. reuteri* TMW1.106 and 100-23ΔgadB accumulated higher levels of glutamate, while *L. reuteri* LTH5448 and 100-23 accumulated GABA (Stromeck et al., 2011; Su et al., 2011). Low levels of GABA in sourdoughs fermented with *L. reuteri* 100-23ΔgadB and *L. reuteri* TMW 1.106 are likely attributable to coelution of peptides or tyrosine during HPLC analysis (Stromeck et al., 2011).

2.3.3 Sensory evaluation of bread by consumer panels

The impact of glutamate on the taste of bread was initially studied with the triangle test and a consumer panel. Comparisons were made between *L. reuteri* TMW1.106 and LTH5448 (accumulating Glu and GABA, respectively); *L. reuteri* 100-23 and 100-23ΔgadB (wild type and isogenic mutant accumulating GABA and Glu, respectively), and *L. reuteri* LTH5448 and 100-23 (both accumulating GABA). With the addition of 3% sourdough and 2% NaCl to bread, the panel did not detect a difference between any of the samples (Table 2-3). Inclusion of 3% sourdough in the bread formula corresponds to a carry-over of about 0.5 mmol glutamate/kg bread.

Table 2-3 Differentiation of sourdough bread produced with different (isogenic) strains of *L. reuteri* by a consumer panel in the triangle test (n=40)

***L. reuteri* strain used for sourdough fermentation**	**Sourdough and NaCl addition to bread (% sourdough / % NaCl flour basis)**			
	6% / 2 %	**6% / 1%**	**6% / 0.5%**	**3% / 2%**
	Number of correct identification (out of 40)			
TMW1.106 vs LTH5448	19	19	21	13
100-23 vs 100-23Δ*gadB*	20	13	16	9
LTH5448 vs 100-23	14	NA	NA	9

Significant differences between the two samples ($p<0.05$) are indicated by gray shading of the respective cells.

With 6% sourdough addition, however, bread produced with *L. reuteri* TMW1.106 and *L. reuteri* LTH5448 as well as bread produced with *L. reuteri* 100-23 and 100-23 ΔgadB differed significantly. In contrast, the taste of breads produced with two GABA-accumulating wild type strains, *L. reuteri* LTH5448 and 100-23, did not differ (Table 2-3). This result suggests that glutamate production in sourdough influences the taste of the bread.

In order to understand the influence of glutamate on the intensity of the salty taste, a consumer panel ranked sourdough breads containing 1% NaCl and control bread containing 1, 1.5, or 2% NaCl according to the intensity of the salty taste (Table 2-4). The consumer panel ranked the salt intensity of the reference bread with 1, 1.5, or 2% in agreement with the salt content of the bread. However, the salty taste intensity of the sourdough bread produced with *L. reuteri* 100-23 or 100-23ΔgadB and at a NaCl level of 1% was ranked equal to the reference bread with 1.5% NaCl and significantly higher than reference bread with the same NaCl content (Table 2-4). Moreover, the intensity of the salty taste of bread produced with *L. reuteri* (1%) was higher when compared to the reference bread with the same level (1%) of NaCl (Table 2-4).

Table 2-4 Ranking of the intensity of the salty taste of bread and sourdough bread and reference bread by a consumer panel (n=39).

Intensity of the salty taste of sourdough bread with 6% sourdough compared to control bread					
Strain # NaCl)	100-23 (1% NaCl)	100-23ΔgadB (1% NaCl)	Control (1% NaCl)	Control (1.5% NaCl)	Control (2% NaCl)
Rank sum	101^{b}	109^{b}	73^{c}	110^{b}	162^{a}
Intensity of the salty taste of sourdough bread with addition of 6% sourdough and 2% NaCl (flour basis) in comparison to control bread with the same NaCl addition					
Strain #	**TMW1.106**	**LTH5448**	**100-23**	**100-23Δ*gadB***	**Control**
Rank sum	114^{a}	124^{a}	123^{a}	120^{a}	74^{b}

Values for the intensity of the salty taste are significantly different if they do not share a common superscript ($p<0.05$).

Sourdough addition thus substantially increased the intensity of the salty taste as perceived by a consumer panel. However, the alternative accumulation of glutamate and GABA during sourdough fermentation did not significantly influence this apparent increase in the salty taste intensity.

2.3.4 Amino acid analysis of bread

The amino acid concentrations in bread produced for evaluation by the trained panel was determined by HPLC. The differences in the amino acid concentrations of sourdough bread produced with the four strains of *L. reuteri* were lower than experimental error, in keeping with the differences in amino acid concentrations in sourdoughs fermented with different strains of *L. reuteri* and the dosage of 6% sourdough in bread (Figure 2-1). The trained panel evaluation compared sourdough breads with three reference breads, a control produced with a straight dough process, a reference including rye malt and gluten, the substrate for sourdough fermentation with *L. reuteri* to control for any influence of the rye malt on sensory properties of bread, and a reference produced with a commercial dried sourdough as benchmark to current industrial practice. The same breads were also analysed with respect to the concentrations of amino acids. The comparison of the amino acid concentration of reference breads and the *L. reuteri* sourdough breads revealed that the amino acid levels in reference breads were generally lower than the corresponding concentrations in sourdough bread fermented with *L. reuteri* (Figure 2-2). Of the reference breads, the highest concentrations were found in bread produced with commercial sourdough while the addition of rye malt did not substantially increase the concentrations of amino acids (Figure 2-2). The glutamate concentration in bread produced with *L. reuteri* 100-23 was about four-fold higher when compared to the straight-dough control and about two fold higher when compared to bread produced with commercial sourdough.

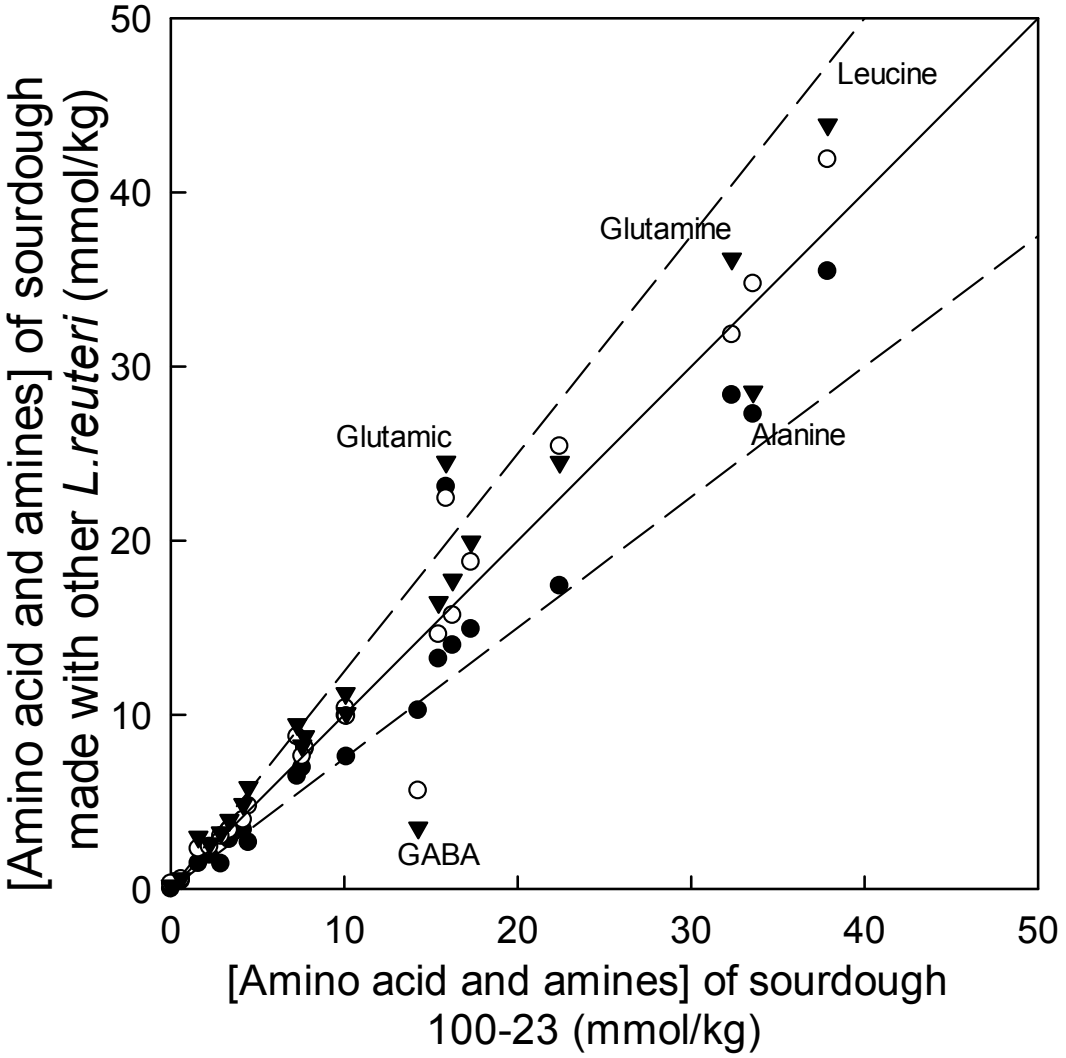

Figure 2-1 Comparison of amino acid concentrations of sourdoughs fermented with four strains of *L. reuteri* after 96 h of fermentation. Amino acid concentrations were plotted against the concentration of the same amino acid in sourdough fermented with *L. reuteri* 100-23; symbol indicate fermentation with *L. reuteri* LTH5448 (●), *L. reuteri* TMW1.106 (▼), and *L. reuteri* 100-23ΔgadB (❍). Data are means of triplicate independent fermentations analyzed in duplicate. The solid line indicates unity; the coefficient of variation was generally less than 25% (indicated by dashed lines). Amino acids of interest are indicated.

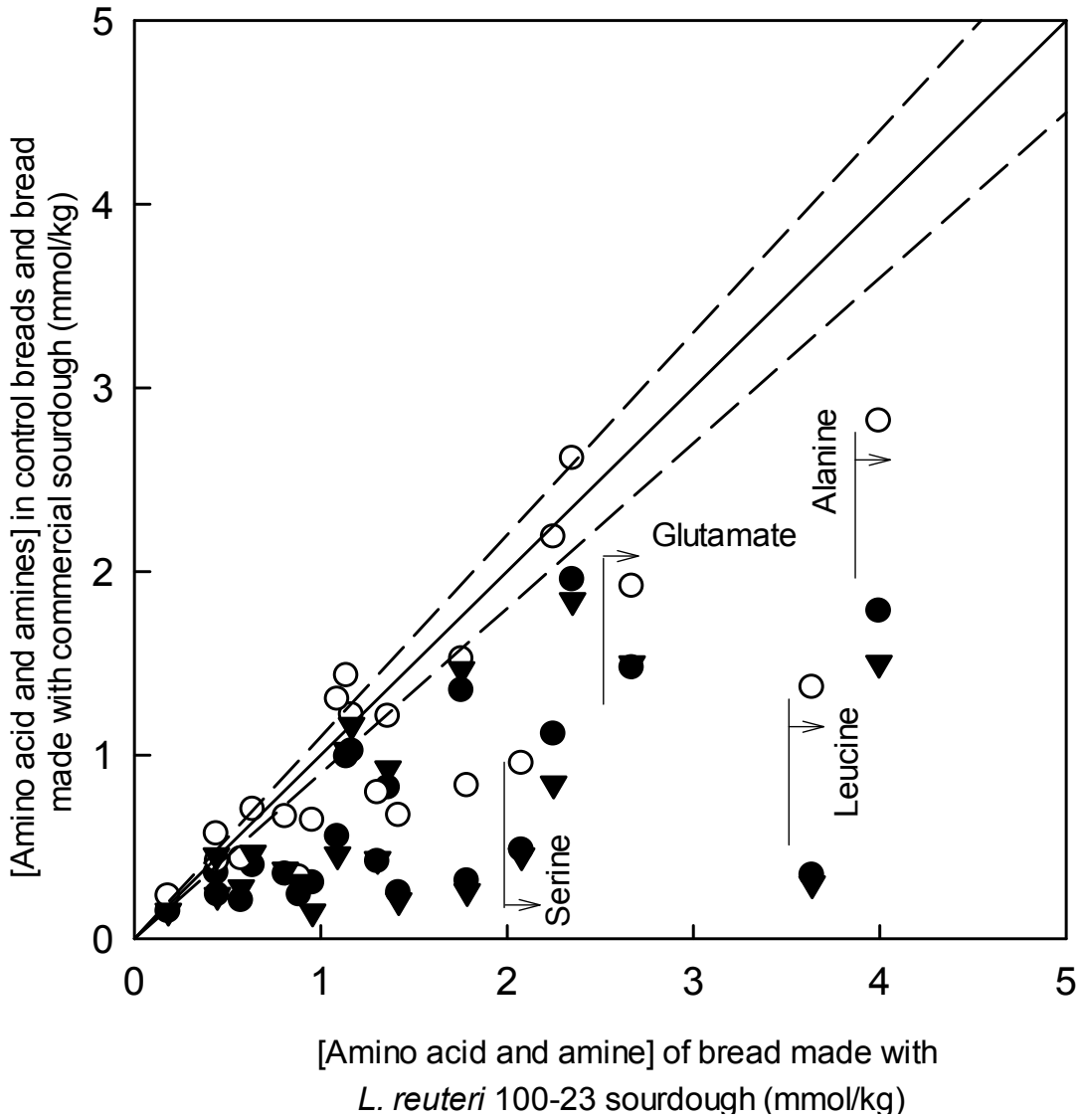

Figure 2-2 Comparison of amino acid concentrations of sourdough bread and reference breads. Amino acid concentrations were plotted against the concentration of the bread produced with *L. reuteri* 100-23 sourdough. Rye malt gluten control (●), commercial sourdough (❍) and regular bread (▼). Amino acid concentrations in bread were analysed with samples from two independent batches of breads and samples were analyzed in duplicate. The solid line indicates unity; the coefficient of variation was generally less than 10% (indicated by dashed lines). Amino acids of interest are indicated.

2.3.5 Sensory evaluation of bread by trained panels

Sourdough breads produced with the addition of 1% and 2% NaCl were evaluated by a trained panel and compared to the three reference breads. The taste threshold of MSG in water and in bread was determined as 0.02% (w/v) in water and 0.03% (w/w) in bread. Figure 2-3 shows the taste intensity of the bread crumb with 1% and 2% salt. The intensity of the salty taste was higher in bread produced with 2% NaCl when compared to bread produced with 1% NaCl but different breads with the same NaCl content had a comparable salty taste intensity (Figure 2-3). Bread with unfermented rye-malt and wheat gluten tasted sweeter than other breads due to the content of sugar and amylase in rye malt (Figure 2-3A and Figure 2-3C). Bread produced with commercial sourdough or *L. reuteri* sourdough had a comparable intensity of sourness; the NaCl content did not influence the sour taste intensity. When compared to reference breads (Figure 2-3A and Figure 2-3C), the intensity of the umami taste was highest in sourdough bread produced with *L. reuteri* 100-23. When breads produced with different strains of *L. reuteri* were compared, sourdough bread produced with *L. reuteri* 100-23ΔgadB consistently exhibited the highest intensity of umami taste and sour taste. The intensity of the umami taste of bread produced with *L. reuteri* 100-23ΔgadB was higher when compared to bread produced with *L. reuteri* 100-23 at a NaCl level of 2% but not at a NaCl level of 1% (Figure 2-3B and Figure 2-3D).

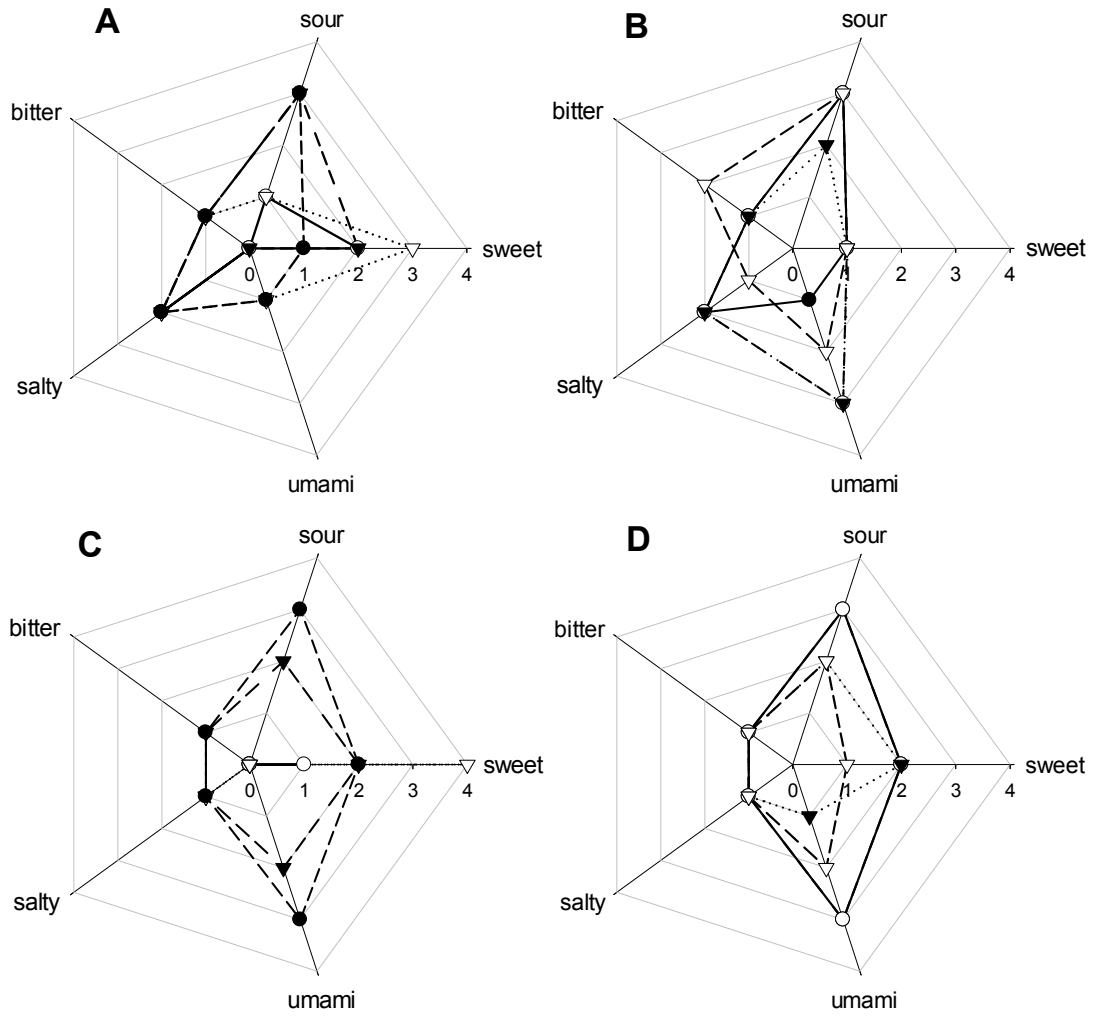

Figure 2-3 Taste profile of the crumb of *L. reuteri* sourdough breads and reference breads as determined by a trained panel with 9 panelists. Panels A and B: bread with 2% NaCl addition (flour basis); Panels C and D: bread with 1% NaCl addition (flour basis). Panels A and C: Reference breads compared to sourdough bread with *L. reuteri* 100-23; regular bread (❍), rye malt gluten control (▽), commercial sourdough bread (▼) and sourdough bread 100-23 (●). Panels B and D: Comparison of sourdough bread produced with different strains of *L. reuteri*: *L. reuteri* 100-23 (●), *L. reuteri* 100-23 ΔgadB (❍), *L. reuteri* LTH5448 (▼) and *L. reuteri* TMW1.106 (▽).

2.3.6 Volume and texture of bread and sourdough bread containing 1 or 2% NaCl.

NaCl addition and acidity influence dough rheology and bread volume. Therefore, pH and acidity as well as the volume and texture of bread were analyzed. The pH, the titratable acidity and the volume of the sourdough bread produced with strains of *L. reuteri* were comparable to bread produced with commercial sourdough. The acidification attained by addition of sourdough was very modest and pH values of sourdough bread ranged from 5.33 to 5.47 (Table 2-5). Reference bread with rye malt and vital wheat gluten had the highest volume at both salt levels due to the addition of wheat gluten (Table 2-5). The bread picture is presented in Appendix A (Figure A-1 and Figure A-2). The crumb stickiness and adhesiveness of the sourdough bread fermented by *L. reuteri* in the same salt level are comparable. The reduction of the NaCl content from 2 to 1% did not result in a consistent decrease in volume or an increase in hardness (Table 2-5); likewise, the inclusion of 6% sourdough in the bread formula did not substantially affect volume or hardness of the bread (Table 2-5).

2.4 Discussion

This study evaluated the effect of glutamate produced during sourdough fermentation on the taste of bread and salt-reduced bread. Sourdough was fermented with wild type strains of *L. reuteri* accumulating glutamate or GABA as alternative end products of glutamine metabolism; moreover, sourdough fermented with the GABA-accumulating *L. reuteri* 100-23 was compared to the isogenic mutant *L. reuteri* 100-23 ΔgadB, which lacks glutamate decarboxylase and thus accumulates GABA (Su et al., 2011). The intensity of the salty taste was assessed with consumer panels and with a trained panel.

Table 2-5 Characterization of acidity, volume, and texture of bread with 1% and 2% salt addition.

	Reference breads			Breads produced with *L. reuteri* sourdough			
Bread with 1% NaCl addition (flour basis)							
	Regular bread	**Commercial sourdough**	**Rye malt gluten**	**TMW1.106**	**LTH5448**	**100-23**	**100-23 Δ*gadB***
pH	6.18±0.00^{a}	5.42±0.01^{b}	6.22±0.35^{a}	5.40±0.02^{b}	5.37±0.01^{b}	5.39±0.03^{b}	5.47±0.03^{b}
TTA	4.06±0.06^{d}	8.23±0.14^{a}	4.35±0.03^{c}	7.89±0.10^{a}	7.96±0.06^{a}	7.72±0.10^{a}	7.54±0.19^{a}
Volume (ml/100g)	182±3 ^{cd}	188±3 ^{bc}	213±3^{a}	181±1^{cd}	177±6^{d}	177±5^{d}	167±4^{e}
Hardness (N)	45±5^{b}	46±4^{b}	15±1^{e}	43±2^{b}	47±5^{b}	48±4^{b}	48±3^{b}
Adhesiveness (g.sec)	34±26^{c}	77±72^{c}	394±98^{a}	28±7^{c}	48±25^{c}	56±34^{c}	57±27^{c}
Crumb stickiness(N)	0.3±0.0^{d}	0.4±0.0^{c}	0.3±0.0^{d}	0.3±0.2^{c}	0.3±0.0^{c}	0.4±0.0^{c}	0.4±0.0^{c}
Bread with 2% NaCl addition (flour basis)							
	Regular bread	**Commercial sourdough**	**Rye malt gluten**	**TMW1.106**	**LTH5448**	**100-23**	**100-23 Δ*gadB***
pH	6.09±0.01^{a}	5.39±0.00^{b}	6.04±0.02^{a}	5.37±0.01^{b}	5.33±0.02^{b}	5.34±0.00^{b}	5.42±0.00^{b}
TTA	3.96±0.05^{d}	8.08±0.01^{a}	4.12±0.03^{c}	7.17±0.22^{b}	7.51±0.09^{ab}	7.57±0.09^{a}	7.36±0.06^{b}
Volume (ml/100g)	190±3^{b}	167±4^{e}	209±5^{a}	178±6^{d}	179±13^{d}	180±10^{d}	175±8^{d}
Hardness (N)	44±3^{bc}	61±4^{a}	21±2^{d}	38±2^{bc}	39±6^{c}	34±2^{c}	39±2^{c}
Adhesiveness (g.sec)	31±12^{c}	52±13^{c}	329±87^{b}	30±8^{c}	37±11^{c}	32±5^{c}	54±14^{c}
Crumb stickiness(N)	1.8±0.5^{ab}	1.8±0.4^{b}	1.4±0.6^{b}	3.2±0.8^{a}	2.9±0.4^{a}	3.5±1.0^{a}	3.0±0.5^{a}

Values in the table for the same parameter (pH, TTA, volume, hardness, adhesiveness, stickiness) differ significantly if they do not share a common superscript ($p<0.05$)

Due to the high level of diverse proteolytic enzymes in germinated rye, gluten proteins are hydrolyzed during the 96 h of fermentation and the amino acid concentrations increase linearly throughout the fermentation (Thiele et al., 2002; Gänzle et al., 2008; Stromeck et al., 2011). Amino acids that are released during sourdough fermentation influence the taste of bread. Glutamate is the major amino acid relevant for umami taste (Zhang et al., 2013*);* the presence of alanine, serine and glycine enhanced the umami taste (Kawai et al., 2002). Glycine, alanine, serine and threonine taste sweet; leucine, isoleucine and phenylalanine

taste bitter (Nishimura & Kato, 1988). Bread produced with lactic acid fermented wheat germ tasted saltier than regular bread (Rizzello et al., 2010); however it remains unclear whether this effect is attributable to organic acid accumulation or the accumulation of specific amino acids.

The taste threshold of MSG in water is 0.03% (Arai et al., 1972). In keeping with this prior report, we determined a taste threshold of MSG in water of 0.02%; the taste threshold of MSG in bread was 0.03%, corresponding to about 2 mmol/kg. The glutamate concentration in rye malt sourdoughs fermented with the four strains of *L. reuteri* was 15 – 25 mmol/kg, about 10 fold higher than the taste threshold in bread. With the addition of 6% sourdough, the concentration of glutamate in sourdough bread produced with *L. reuteri* matches approximately the threshold of MSG in bread; however, the glutamate concentration in reference breads, including bread produced with a commercial sourdough preparation, was below the taste threshold. Accordingly, sourdough bread produced with *L. reuteri* had an overall higher umami taste than the reference bread. The umami taste intensity of bread produced with *L. reuteri* 100-23ΔgadB was equal or higher than any other bread evaluated in this study. These differences in the umami taste between breads are remarkable because the difference in the glutamate concentrations between sourdough breads was calculated to be equal or less than the taste threshold of glutamate (1 – 2 mmol/kg). All sourdough breads including the reference produced with commercial sourdough tasted sourer than other breads.

The trained taste panel reported a comparable level of saltiness in all breads while the consumer panel judged sourdough breads produced with *L. reuteri* as tasting saltier. Trained panels are appropriate to analyze the attributes, while consumer panels are

conducted to assess acceptability, preference and/or difference (Hough et al., 2002). Sensory analysis with trained panels in combination with consumer scores may identify and define the contribution of taste attributes to the overall consumer acceptance of the product (Meilgaard et al., 1999). Previous reports indicated that rye bread with a higher level of acidity allowed NaCl reduction without affecting the perceived saltiness and pleasantness (Hellemann, 1992; Tuorila-Ollikainen, et al., 1986). Likewise, the addition of umami compounds to soup maintained the palatability despite sodium reduction (Yamaguchi & Takahashi, 1984). Taken together, this study demonstrates that sourdough fermentation increases the umami and sour taste intensity of bread; this overall increased taste intensity compensates for lower saltiness in NaCl-reduced bread. In contrast, the bland taste of bread produced with a straight dough process requires higher salt addition to achieve a comparable perceived saltiness and pleasantness. Sourdough addition had no substantial influence on the texture or the volume of bread. Improved volume and texture of sourdough bread may be achieved by sucrose supplementation of sourdough to support synthesis of exopolysaccharides (Kaditzky et al., 2008; Rizzello et al., 2010).

In addition to the accumulation of taste-active compounds, sourdough fermentation with *L. reuteri* accumulates ornithine, which enhances the roasty flavor of the bread crust (Thiele et al., 2002; Stromeck et al., 2011). Antifungal compounds produced during sourdough fermentation may prolong the mould-free shelf life of bread (Black et al., 2013; Rizzello et al., 2010). Sourdough and specifically rye-malt sourdoughs fermented with *L. reuteri* also contain anti-hypertensive peptides and GABA (Coda, et al., 2012; Hu et al., 2011).

2.5 Conclusion

The simultaneous accumulation of organic acids and glutamate to allow NaCl reduction and protein metabolites with a potential antihypertensive effect makes sourdough a promising ingredient for development of bread with improved nutritional and sensory properties.

3 Synthesis of γ-glutamyl dipeptides during sourdough fermentation by *L. reuteri*

3.1 Introduction

Kokumi active compounds impart long lasting mouthfulness, complexity and continuity of taste (Roudotalgaron et al., 1994; Toelstede & Hofmann, 2008a). In contrast to the six basic tastes, kokumi do not directly interact with taste receptors but enhance the perception of other taste compounds (Running et al., 2015). Kokumi taste activity relates to the interaction of the calcium-sensing receptor (CaSR) (Ohsu et al., 2010). Kokumi peptides, such as γ-Glu-Cys-Gly, γ-Glu-Val, γ-Glu-Ala, γ-Glu-Cys, and γ-Glu-Val-Gly are CaSR agonists. Thus, they do not interact with the taste receptors themselves but modulate signal transduction from taste receptors to the brain (Kuroda & Miyamura, 2015; Ohsu et al., 2010).

Kokumi peptides were isolated from ripened Gouda cheese, beans, yeast extract, and soy sauce (Kasai et al., 1983; Liu et al., 2014; Roudotalgaron et al., 1994; Toelstede & Hofmann, 2008a; Toelstede & Hofmann, 2008b; Toelstede et al., 2009). Addition of 0.004% of γ-Glu-Val-Gly in chicken soup and reduced-fat cream significantly increases the thickness of taste and the intensity of aftertaste (Kuroda & Miyamura, 2015). The intensity of sweetness, saltiness and umami were increased in presence of a kokumi compound (Kuroda & Miyamura, 2015). Omission and reconstitution experiments in aqueous solution and in a cheese matrix confirmed that the γ-glutamyl dipeptides, such as γ-Glu-Glu, γ-Glu-Leu, γ-Glu-Ile, γ-Glu-Val, γ-Glu-Tyr, γ-Glu-Gln, γ-Glu-Lys impart the kokumi sensation (Liu et al., 2014; Toelstede et al., 2009). Glycopeptides and γ-glutamyl tripeptides also impart kokumi sensation (Miyamura et al., 2015; Roudotalgaron et al., 1994; Ueda et al.,

1997; Iwasaki et al., 2009). In contrast, α-glutamyl dipeptides, which do not have kokumi sensation, can impart umami taste.

Formation of kokumi peptide in soy sauce and cheese is attributed to microbial activity. The synthesis of γ-glutamyl peptide might be attributed to the glutathione degradation or the conversion of glutamine by γ-glutamyl transferase (GGT). GGT is found in *Penicillium* in cheese, and in *Aspergillus* or *Bacillus* from soy sauce. Quantitative analysis of glutamyl dipeptides in ripened Gouda cheese revealed that ripening increased the level of kokumi active γ-glutamyl dipeptides (Toelstede et al., 2009). Different from α-peptide bond, γ-glutamyl peptides had higher solubility in water and were also resistant to hydrolysis by peptidases (Suzuki & Kumagai, 2004b). The proteolysis of casein only yields α-bonds; therefore, the presence of γ-glutamyl peptides can be attributed to the action of γ-glutamyl transferase and/or γ-Glu-Cys synthetase from microbial activity (Roudotalgaron et al., 1994). Recent studies indicated that some *Lactobacillus* might produce γ-glutamyl peptide from free amino acids but their contribution is still unclear (Arai et al., 1973; Sgarbi et al., 2013; Toelstede & Hofmann, 2009).

The type II sourdough has been used as baking improver to enhance the bread taste and texture. During sourdough fermentation, cereal enzymes and LAB generate free amino acids, fatty acids, EPS, acetic acid and decrease pH, which contribute to the bread quality (Gänzle et al., 2007; Gänzle et al., 2008). Sourdough fermented with isogenic glutamate accumulating *L. reuteri* 100-23ΔgadB had higher intensity of umami taste than sourdoughs fermented with GABA producing *L. reuteri* 100-23 due to the different glutamate levels (refer to Chapter 2). Comparison of sourdough fermented with *L. reuteri* wild type and

mutant exclude the effect of acidity and confirm the contribution of glutamate (refer to Chapter 2). But the effect of peptides, especially kokumi active peptides is still unclear.

The aim of this work is to identify and quantify γ-glutamyl dipeptides from sourdough fermented by different strains of *L. reuteri* in order to study the synthesis pathway. Besides, the concentration of γ-glutamyl dipeptides related to the taste of sourdough bread will be investigated.

3.2 Materials and methods

3.2.1 Strains and growth conditions

L. reuteri TMW1.106, LTH5448, 100-23, 100-23ΔgadB were grown in modified de Man, Rogosa and Sharpe Medium (mMRS) at 37°C and *L. sakei* LS8 was grown at 32°C. Inoculum for fermentation was prepared by harvesting of cells from overnight cultures in mMRS at 4000×g for 5 min. Cells were washed twice in sterile tap water and resuspended in tap water to the initial culture volume. For the sourdough prepared for sensory evaluation, strains were grown in 10% food grade wort for 24 h (CBW Munich pure malt extract, BRIESS malt&ingredients Co., Chilton, USA). Strains were subcultured twice and used for sourdough fermentation without washing.

3.2.2 Materials and chemicals

Transglutaminase was obtained from Ajinomoto (Tokyo, Japan). Six kokumi dipeptide standards (γ-Glu-leu, γ-Glu-Ile, γ-Glu-Glu, γ-Glu-Met, γ-Glu-Val, γ-Glu-Phe) were obtained from United BioSystems Inc (Herndon, VA, USA). Fungal protease from *Aspergillus oryzae* was obtained from Sigma-Aldrich (St.Louise, MO, USA). Food grade

Flavourzyme for the sensory evaluation was obtained from Lallemand Baking solutions (Anjou, QC, Canada).

3.2.3 Sourdough fermentation with different ingredients

In order to understand the effect of the raw material, sourdoughs were fermented with or without fungal protease, with or without microtransglutaminase, rye malt or rye flour by *L. reuteri* 100-23 and *L. reuteri* 100-23 ΔgadB for 0 h, 48 h and 96 h respectively. The pH and cell counts were determined in fresh sourdough. Freeze-dried sourdoughs were used for LC-MS to identify the γ-glutamyl dipeptides and free amino nitrogen analysis. All fermentations were carried out in two independent experiments and samples were analysed in duplicate. Results are reported as means ± standard deviation.

3.2.4 Sourdough fermented by different strains of *L. reuteri*

Rye malt flour was obtained from Laihian Mallas (Laihia, Finland) and vital gluten was obtained in a local supermarket. Cultures of *L. reuteri* (4 ml) were mixed with 0.5 g rye malt and 0.5 g vital gluten and fermented at 37°C for 24, 48, 72 and 96 h. Chemically acidified dough was prepared using an acetic acid: lactic acid (1:4, v/v) mixture to mimic the acidification by *Lactobacillus* and the final dough pH was 3.5±0.25. Fresh samples were used for pH, cell counts and γ-glutamyl dipeptides analysis by HPLC-MS/MS. Freeze dried samples were used for free amino nitrogen analysis. Sourdough was prepared in two independent fermentations and samples were analysed in duplicate. Results are reported as means ± standard deviation.

3.2.5 Synthesis of γ-glutamyl peptides during buffer fermentation

L. reuteri 100-23 and *L. reuteri* LTH5448 were grown in mMRS overnight and washed twice by autoclaved tap water. An aliquot of this culture was mixed with 5 g/L of maltose and 10 mmol/L of lysine, methionine, glutamine, glutamate, leucine, isoleucine, phenylalanine and valine at 37 °C at pH 6.5. Samples were collected at 0, 24, 48, 72 and 96 h for LC-MS/MS to analyze γ-glutamyl dipeptides. Controls inoculated with strains and maltose but without any additional amino acids, or with maltose and amino acids solution but without any strain inoculation were also prepared. The cell count and pH were determined on each sample. All these experiments were carried out in two independent fermentations and samples were analyzed in duplicate.

3.2.6 Bread baking

The bread used for evaluation by a consumer panel was baked at the University of Alberta food kitchen. The cultures of *L. reuteri* (50 ml) were mixed with 50 g white wheat flour and fermented at 37°C for 24 h. Then 75 g rye malt flour and 75 g wheat gluten, were mixed with 350 ml water and fermented at 37°C for 72 h. The control sourdough was fermented by 50 ml *L. sakei* with 50 g wheat flour for 24 h at 32°C. Samples were collected immediately for pH, cell count and HRM-qPCR. Sourdough was freeze dried and stored at room temperature for the future baking trial for sensory evaluation. Regular flour was replaced by 5% dried sourdough and mixed with 2% sugar, 2% yeast, 1.5% or 2% salt and 70% water in a spiral kneader (Kitchen Aid K45SS, Hobart Co. Troy, OH, USA) for 5 min. The dough was rest for 2 h at 30 °C in a proofer followed by dough shaping and 1 h proof (Res-Cor, Crescent Metal Products Inc, Cleveland, OH, USA). Bread was baked in a multi-deck oven (Bakers Pride, Lachine, QC, Canada) with forced air set at a temperature of

210 °C for 25 min. The bread were cooled down for 2 h at room temperature and then packed in labeled and sealed polyethylene bags and frozen at -18°C for sensory evaluation.

3.2.7 DNA isolation and HRM qPCR identification

The identity of the sourdough fermentation microbiota with the inoculum was verified by high resolution melting quantitative PCR as described previously (Lin & Gänzle, 2014). DNA was isolated from 1.0 mL fresh sourdough or overnight cultures of *L. reuteri* LTH5448 and 100-23, and *L. sakei* LS8 grown in mMRS broth using a DNeasy Blood and Tissue kit according to the instructions of the manufacturer (Qiagen, Mississauga, ON, Canada). HRM-qPCR (Rotor-GeneQ, Qiagen, Mississauga, ON, Canada) was used to amplify bacterial 16S rDNA.

3.2.8 Sensory consumer panel

The sensory studies were reviewed for their adherence to ethical guidelines and approved by the Research Ethics Board at the University of Alberta.

The frozen bread was thawed 24 h at room temperature before sensory analysis. The bread crust was cut off and the breadcrumb was cut into 1 cm^3 pieces. The samples were placed in separate covered petri dishes labeled with 3-digit random numbers and 3 pieces were presented to the panelists. Sensory evaluation was performed in the sensory testing laboratory at the Department of Agricultural, Food and Nutritional Science, University of Alberta.

The panelists were recruited randomly at the Agricultural & Forestry Centre, University of Alberta. The number of the male and female panelist was about equal. Most of the panelist (78%) were 18-29 years old. Majority of the panelists consumed bread more than

2-3 times per week and indicated that they like bread. Over 60% of the panelists think taste is the most important attribute compared to flavor, texture and color.

The panelists received encoded samples and questionnaire, as well as instructions for the evaluation of samples. Samples were randomly assigned to each panelist. The samples were presented to the assessors blind, so that the panelists did not know which sample they were evaluating. Water was provided to cleanse the palate between samples. The preference and paired comparison on salty taste were used for 1.5% and 2% salt level bread as described in Chapter 2. The number of correct responses and the number of total responses were counted. The Just About Right (JAR) test was used to evaluate the saltiness and aftertaste of the bread fermented by *L. reuteri* 100-23 and *L. reuteri* LTH5448 at 2% salt level. The frequency of each category was calculated. The intensity of saltiness of *L. reuteri* 100-23, *L. reuteri* LTH5448, *L. sakei* LS8 and regular bread at 1.5% salt level was evaluated by ranking test. The sum of the rank for each sample was calculated and the result was analyzed by Friedman test (Takahashi et al., 2002).

3.2.9 Free amino nitrogen

Free amino nitrogen content of the SDS-soluble fraction in different samples was measured by the ninhydrin method. Freeze-dried samples (50 mg) were suspended in 1 mL sodium phosphate buffer (200 mmol L^{-1}) and incubated at 23°C with agitation (250 rpm) for 1 h. Solids were removed by centrifugation 10 min at 10,000 x *g*; 10 µL of supernatant was mixed with 190 µL of phosphate buffer and 100 µL of ninhydrin solution (5.0 g Na_2HPO_4, 6.0 g of KH_2PO_4, 0.3 g of fructose and 0.5 g of Ninhydrin) (Sigma-Aldrich, USA) in 100 mL of Milli-Q water at pH 6.7 and incubated at 100°C for 16 min. After cooling down at room temperature for 20 min. It was mixed with 500 ul of KIO_3 solution

(0.2 g KIO_3 dissolved in 60 mL of distilled water and 40 mL of 96% ethanol) before measuring at 570nm (Jasco, Gross-μmolstad; Germany). Glycine solution with concentrations from 2.0 to 20.0 mg L^{-1} was used to establish a calibration curve.

3.2.10 Quantification of kokumi peptides by LC-MS/MS analysis

Peptide quantification was performed using a 1200 series HPLC unit and diode array detector (DAD) (Agilent Technologies, Palo Alto, CA, USA) connected to a 4000 Q TRAP LC-MS/MS System (MDS SCIEX, Applied Biosystems, Streetsville, ON, Canada). Peptides were separated on a Luna C18 RP-HPLC column (5 um, 250 mm X 4.6 mm, Phenomenex, Torrance, CA, USA) and detected from 190 to 400 nm. Mobile phase A consisted of 0.1% formic acid in Milli-Q water. Mobile phase B consisted of 0.1% formic acid in acetonitrile. Samples were eluted at a flow of 0.5 ml min^{-1} with the following gradient: 4-16 min, 100%A; 16-20 min, 100-95%A; 20-30 min, 95-75%A; 30-45 min, 75-65%A, 45-47 min 65-0%A; 47-57 min, 0%A; 57-67 min, 100%A; and re-equilibration time of 10 min.

LC-MS/MS analysis was performed using atmospheric pressure electrospray ionization in positive mode. The protonated precursor ions and the dominant fragments of each of the six-kokumi peptides were optimized. The samples were detected and quantified using multiple reaction monitoring mode (MRM). LC-MS/MS parameters for quantification of the six kokumi peptides are shown in Table 3-1. The values for optimum ion source parameters were as follows: spray voltage 4 KV, collision energy 10, curtain gas 10, and declustering potential at 20 V. Data acquisition was interfaced to a computer workstation running Analyst 1.5 (Applied Biosystems, USA). External calibration standards (0.005 to

50 mg/L) of γ-glutamyl dipeptides were prepared at 30% (v/v) methanol in 0.1% aqueous formic acid. The limit of quantification is 0.5 mg/L.

Table 3-1 LC-MS/MS parameters for the determination of six γ-glutamyl dipeptides in water-soluble extracts of sourdough. The kokumi taste threshold of γ-glutamyl dipeptides were summarised from other studies (Toelstede et al., 2009; Toelstede & Hofmann, 2009).

γ-glutamyl peptides	Transition	Retention time (min)	Taste threshold (μmol/kg)
γ-Glu-Val	247.4→230.6	31.1	3.3
γ-Glu -Met	279.6→133.6	32.2	5
γ-Glu -Glu	276.7→260.0	6.8	17.5
γ-Glu -Leu	261.4→244.6	37.5	5
γ-Glu -Phe	295.8→166.3	40.2	2.5
γ-Glu -Ile	261.4→244.6	36.7	Not available

3.2.11 Statistical analysis

Statistical analysis of concentration of γ-glutamyl dipeptides and free amino nitrogen of sourdough was performed by analysis of variance (ANOVA) with the procedure PROC GLM using the Statistical Analysis System V.9.2 (SAS Institute Inc., Cary, NC, USA). Values for peptide concentrations or level of free amino nitrogen were considered significantly different at a 5% error level ($P < 0.05$).

3.3 Results

3.3.1 Characterization of sourdoughs

Sourdough was fermented with different raw materials and with different strains from 0-96 h. All colonies obtained from sourdoughs exhibited a uniform colony morphology matching the inoculum. HRM-qPCR with template DNA from sourdoughs generated

amplicons with a size and melting temperature (86.5°C) matching the amplicons from *L. reuteri* (data not shown). Cell counts and pH were comparable with results previously obtained with the same strains (Appendix Table B-1) (Chapter 2). pH and cell count of sourdoughs fermented with different raw materials or different strains not show any significant difference, excluding the effect of the environment stress on different strains. Chemically acidified doughs maintained a constant pH of 3.4-4.0 through out the incubations and the cell counts were below 10^4 cfu ml^{-1}.

3.3.2 Sensory evaluation of salty taste in 3 types of bread

To understand the effect of long-term fermentation on the taste of bread, the sensory evaluation of sourdough bread fermented with *L. reuteri* for 72 h were compared. In order to control the effect of acidity and pH, sourdough fermented with *L. sakei* was used as reference.

Table 3-2 Ranking test of salty taste of 3 types of bread with 1.5% salt level. (n=42)

	***L. reuteri* 100-23**	***L. reuteri* LTH5448**	**Regular**	***L. sakei***
Rank average	142[a]	132[a]	67[b]	79[b]

The consumer panel ranked the salty intensity of the bread fermented with *L. sakei* (type I), regular bread without sourdough addition, and sourdough bread fermented with *L. reuteri* LTH5448 and *L. reuteri* 100-23 (type II) at 1.5% salt level (Table 3-2). The salty taste intensity of sourdough bread produced with *L. reuteri* 100-23 and *L. reuteri* LTH5448 was ranked higher than *L. sakei* (type I) bread and regular bread. The acidity is not related to the taste difference since pH of sourdough fermented after 24 h was comparable (Chapter 2). Therefore, several amino acids and dipeptides accumulated during fermentation may

affect salty taste. It is interesting to see that there is no significant difference in salty taste intensity between breads prepared with *L. reuteri* 100-23 and *L. reuteri* LTH5448.

3.3.3 Effect of the raw material and other ingredients on the γ-glutamyl dipeptides during sourdough fermentation

To determine whether γ-glutamyl dipeptides accumulate during sourdough fermentation, and to provide initial insights into the pathways for their formation, rye sourdoughs were fermented with different combinations of rye malt, protease, and transglutaminase with *L. reuteri* 100-23 and *L. reuteri* 100-23 ΔgadB. Overall, the free amino nitrogen (FAN) of sourdough fermented for 96 h is significantly higher than that of sourdough fermented for 48 h (Figure 3-1). Sourdough fermented with fungal protease and rye malt shows the highest FAN compared to all the other sourdoughs with the same incubation time indicating that fungal protease determined the proteolysis (Figure 3-1). However, there was no significant difference between sourdoughs fermented with rye malt or rye flour, with or without transglutaminase. This indicates the enzyme from rye malt and transglutaminase are not the most important contributors to the proteolysis (Appendix Table B-2).

About 11 γ-glutamyl peptides impart kokum sensation with known threshold and 9 are γ-glutamyl dipeptides (Toelstede t al., 2009; Toelstede & Hofmann, 2009). Among those 9 γ-glutamyl dipeptides, 6 were selected in this research corresponding to the amino acids composition of cereal proteins. During sourdough fermentation, γ-glutamyl dipeptides were produced and the high levels corresponded to high FAN level (Figure 3-1,

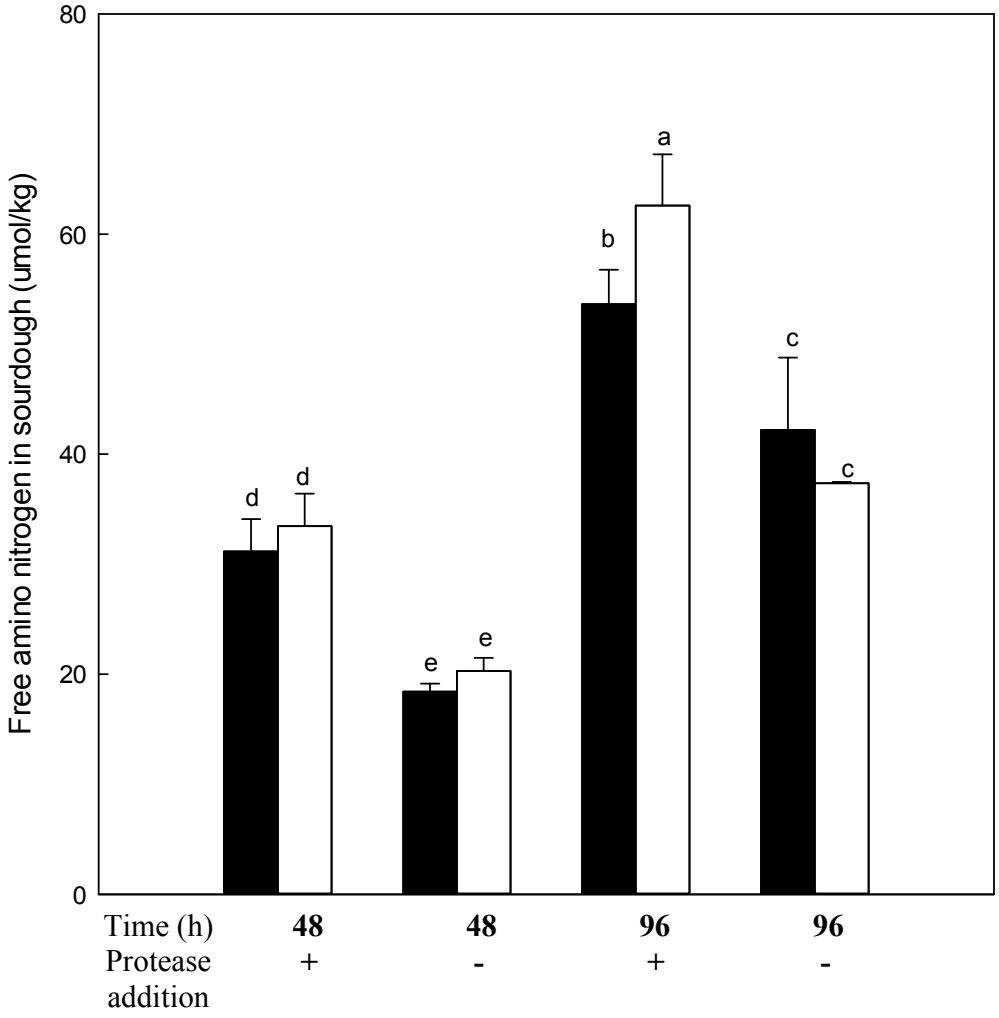

Figure 3-1 Free amino nitrogen of different type of sourdough fermented by *L. reuteri* 100-23 (black bar) and *L. reuteri* 100-23ΔgadB (white bar) for 48 h and 96 h respectively. The different letter in the same figure means significant difference ($p<0.05$).

Table 3-3). There is no difference between *L. reuteri* 100-23 and100-23 ΔgadB, which only differ in the level of glutamate and γ-aminobutyric acid (GABA) (Su et al., 2011). Also, the rye malt flour and transglutaminase did not affect the level of γ-glutamyl dipeptides and FAN (Appendix Figure B-1). γ-Glu-Glu, γ-Glu-Leu and γ-Glu-Ile are the major accumulated γ-glutamyl dipeptides whereas γ-Glu-Met, γ-Glu-Phe, and γ-Glu-Val are very

low in all the samples (Table 3-*3*). This may relate to the amino acid composition of cereals, that glutamine, glutamate, leucine, and isoleucine are the major amino acids in wheat and rye (Tkachuk & Irvine, 1969). Overall, γ-glutamyl peptide accumulateion in sourdough without fungal protease were generally lower than that in all the other sourdoughs, whereas sourdough fermented with fungal protease and rye malt accumulated overall higher γ-glutamyl dipeptides than the others (Table 3-*3*). The LC/MS and FAN results demonstrated that the level of γ-glutamyl peptides were higher in sourdough with higher FAN and proteolytic activity. Fungal protease plays an important role in the production of γ-glutamyl dipeptides during sourdough fermentation.

Table 3-3 Concentration of γ-glutamyl dipeptides (μmol/kg) in sourdough fermented with different ingredients (n=2).

	Glu-Glu	**Glu-Ile**	**Glu-Leu**	**Glu-Met**	**Glu-Phe**	**Glu-Val**
			L.reuteri 100-23 0 h			
Rye malt+ Fungal protease	7.36±2.59 ^{d}	1.57±1.57^{bc}	3.04±3.04	1.68±0.00^{f}	NA	NA
Rye malt	9.59±0.44 ^{c}	2.05±0.18 ^{c}	3.05±3.05	1.69±0.01^{f}	NA	0.35±0.49
			L.reuteri 100-23 48 h			
Rye malt+ Fungal protease	14.50±1.11 ^{b}	7.18±0.92 ^{a}	3.72±1.17	5.10±0.46 ^{a}	NA	1.74±1.20
Rye malt	9.68±0.49 ^{c}	1.76±0.66^{bc}	4.50±0.23	4.24±0.25 ^{b}	NA	1.25±0.09
			L.reuteri 100-23 96 h			
Rye malt+ Fungal protease	10.17±1.19 ^{c}	2.46±0.68^{c}	4.31±0.02	4.49±0.20 ^{b}	0.01±0.00	4.60±0.57
Rye malt	9.83±1.88 ^{c}	NA	2.26±0.96	3.24±0.33 ^{c}	NA	1.58±2.51
			L.reuteri 100-23 ΔgadB 0 h			
Rye malt+ Fungal protease	8.71±0.66 ^{c}	1.55±0.41 ^{bc}	3.36±3.36	1.68±0.00 ^{f}	NA	1.17±1.66
Rye malt	9.08±0.51 ^{c}	5.17±0.33 ^{a}	2.63±2.63	1.69±0.01 ^{f}	NA	0.29±0.41
			L.reuteri 100-23 ΔgadB 48 h			
Rye malt+ Fungal protease	10.25±3.31^{c}	5.69±3.11^{a}	6.53±0.32	3.37±1.03^{c}	NA	0.27±0.56
Rye malt	10.45±0.86^{c}	0.20±0.20^{c}	6.60±0.80	1.64±0.01 ^{f}	NA	1.62±0.14
			L.reuteri 100-23 ΔgadB 96 h			
Rye malt+ Fungal protease	14.85±1.19^{b}	2.65±1.05^{b}	5.22±1.57	3.17±0.85^{c}	0.01±0.00	1.04±0.75
Rye malt	12.64±3.14^{b}	NA	4.25±0.00	2.15±0.67^{e}	NA	NA

[a]The different letter in the same column means significant difference.

3.3.4 Screening and identification of γ-glutamyl dipeptides in sourdough

Peptidase activity and accumulation of amino acids and peptides by strains of *L. reuteri* are strain specific (Hu et al., 2011). To determine the strain specificity on the synthesis of γ-glutamyl peptides, sourdoughs were fermented with different strains of *L. reuteri*. The FAN increased about 2 fold after 72 h compared to the corresponding level at 24 h (Figure 3-2). *L. reuteri* LTH5448 and *L. reuteri* 100-23 at 72 h had the highest level of free amino nitrogen compared to all the other samples. Overall, there is no significant difference between the strains in the same fermentation time (Figure 3-2). The free amino nitrogen contents were generally higher in lactobacilli-fermented sourdoughs as compared to the corresponding chemically acidified dough at the same fermentation time (Figure 3-2).

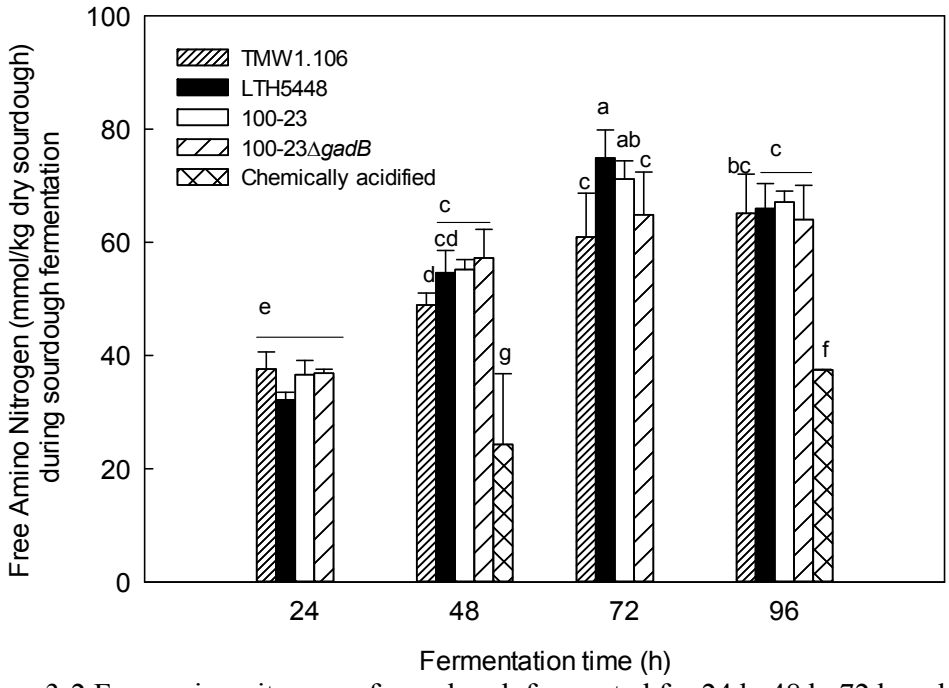

Figure 3-2 Free amino nitrogen of sourdough fermented for 24 h, 48 h, 72 h and 96 h by *L. reuteri* TMW 1.106, *L. reuteri* LTH5448, *L.reuteri* 100-23 and *L. reuteri* 100-23 *Δ*gadB respectively. The chemically acidifed dough fermented for 48 h and 96 h were also evalauted. The different letters indicate significant difference ($p<0.05$).

Table 3-4 Concentration of γ-glutamyl dipeptides (μmol/kg) in sourdough fermented from 0 to 96 h by different strains (n=2).

	γ-Glu-Glu	γ-Glu-Ile	γ-Glu-Leu	γ-Glu-Met	γ-Glu-Phe	γ-Glu-Val
			0 h			
TMW1.106	2.8±0^{d}	1.4±0.3^{d}	1.9±0.6	1.4±0.2^{e}	1.2±0.9	1.1±0.2^{g}
LTH5448	7.6±2.2^{d}	6.2±2.1^{b}	2.9±0.1	1.3±0.0^{e}	2.1±0.0	1.1±0.1^{g}
100-23	7.2±0.3^{d}	1.4±0.1^{d}	1.9±0.4	1.3±0.0^{e}	1.2±0.5	1.8±0.8^{g}
100-23Δ*gadB*	4.5±0.6^{d}	1.2±0.0^{d}	1.7±0.2	1.3±0.0^{e}	1.0±0.2	1.3±0.4^{g}
Chemically acidified	2.8±0^{d}	0.3±0.0^{e}	0.7±0.0	1.4±0.0^{e}	3.8±0.7	1.3±0.5^{g}
			24 h			
TMW 1.106	14.8±4. 4^{c}	2.6±0.2^{c}	3.6±0.3	3.6±0.2^{c}	1.3±0.2	15.7±2.4^{f}
LTH5448	13.3±1.0^{c}	17.0±0.6^{a}	5.8±0.1	4.5±0.2^{b}	1.2±0.0	26.5±0.5^{e}
100-23	17.3±2.0^{bc}	2.9±0.1^{c}	4.3±0.1	4.2±0.5^{b}	1.2±0.0	16.4±0.3^{f}
100-23Δ*gadB*	18.2±1.7^{bc}	2.4±0.2^{c}	3.8±0.2	3.5±0.1^{c}	1.2±0.1	18.2±1.5^{f}
Chemically acidified	6.2±2.3^{d}	1.2±0.1^{d}	3.9±0.1	1.9±0.0^{e}	1.0±0.1	2.3±0.0^{g}
			48 h			
TMW 1.106	15.5±0.9^{c}	2.9±0.2^{c}	4.1±0.5	3.6±0.2^{c}	1.2±0.1	27.4±2.0^{e}
LTH5448	20.6±1.9^{bc}	16.9±0.7^{a}	5.6±0.1	4.0±1.2^{d}	1.3±0.1	52.0±1.0^{c}
100-23	13.1±0.4^{cd}	2.6±0.2^{c}	4.4±0.0	3.4±0.5^{c}	1.2±0.0	29.7±1.9^{e}
100-23Δ*gadB*	20.2±0.4^{bc}	2.5±0.3^{c}	4.4±0.4	3.9±0.1^{bc}	1.1±0.0	35.4±3.1^{d}
Chemically acidified	11.9±2.8^{d}	0.8±0.1^{e}	7.5±0.5	2.7±0.2^{e}	na	2.8±0.3^{g}
			72 h			
TMW1.106	13.3±1.4^{c}	2.9±0.4^{c}	5.0±0.0	3.7±0.3^{bc}	1.4±0.1	39.0±4.5^{d}
LTH5448	12.6±1.9^{cd}	16.5±0.3^{a}	6.7±0.1	3.2±0.2^{cd}	1.4±0.1	74.0±5.0^{a}
100-23	13.5±1.5^{c}	2.7±0.0^{c}	5.1±0.1	3.3±0.5^{c}	1.4±0.2	40.4±7.9^{d}
100-23ΔgadB	14.9±0.5^{c}	2.4±0.0^{c}	4.3±0.2	3.3±0.5^{c}	1.2±0.0	34.4±3.1^{d}
Chemically acidified	5.8±1.1^{d}	0.9±0.1^{e}	5.5±0.1	1.9±0.0^{e}	1.2±0.0	2.3±0.3^{g}
			96 h			
TMW 1.106	22.0±6.5^{b}	1.6±0.1^{d}	8.2±0.7	3.9±0.0^{bc}	3.1±0.1	37.5±1.1^{d}
LTH5448	20.2±7.0^{bc}	7.0±1.0^{b}	9.2±2.7	4.2±1.0^{b}	3.5±1.5	67.5±1.5^{b}
100-23	28.9±14.5^{a}	2.5±0.5^{c}	7.7±1.1	4.2±1.2^{bc}	3.4±0.8	36.5±0.4^{d}
100-23Δ*gadB*	29.0±13.3^{a}	1.7±0.3^{d}	7.8±0.3	5.1±0.0^{a}	3.6±0.5	48.2±3.6^{c}
Chemically acidified	7.1±0.7^{d}	1.4±0.2^{b}	6.7±0.2	1.8±0.5^{e}	1.6±0.2	2.7±0.3^{g}

The content of γ-glutamyl dipeptides in lactobacilli-fermented sourdough is overall higher than that in chemically acidified dough (Table 3-4). The extended fermentation time is beneficial for the accumulation of γ-glutamyl dipeptides in general. Among sourdoughs, *L.*

reuteri LTH5448 accumulated higher amount of γ-Glu-Ile and γ-Glu-Val whereas *L. reuteri* 100-23 ΔgadB and *L. reuteri* 100-23 generated higher amount of γ-Glu-Glu and γ-Glu-Met (Table 3-4). A large difference between strains and peptide composition is noticed. This indicated that proteolysis is important, but the strain specificity also determined γ-glutamyl peptides synthesis.

3.3.5 Generation of γ-glutamyl peptides in buffer fermentation

To confirm whether the *L. reuteri* is able to produce the γ-glutamyl peptides, the buffer fermentation was used with glutamine (donor amino acid) and a mixture of the candidate acceptor amino acids, including Glu, Leu, Met, Val, Lys, Ile, Phe in the presence of maltose at 37°C for 0-96 h. The content of γ-glutamyl dipeptides in the controls, with cultures, maltose but without amino acids or with maltose, amino acids but without cultures, were below the detection limit. The pH and cell count were analyzed after the sample collection (Table 3-5). The cell lysed after 24 h in the buffer fermentation and not further increase the level of peptides (data not shown). Similar to sourdough, the synthesis of γ-glutamyl

Table 3-5 pH and cell count of the amino acids model fermented by *L. reuteri* LTH5448 and 100-23 from 0 h to 24 h.

	LTH5448	100-23	LTH5448 control	100-23 control
		pH		
0 h	6.53±0.01	6.81±0.05	6.42±0.00	6.59±0.00
24 h	4.78±0.00	4.61±0.00	4.59±0.01	4.53±0.00
		Cell count		
0 h	6.20E+08	5.20E+08	6.00E+08	4.20E+08
24 h	1.44E+08	1.80E+08	2.02E+08	4.96E+07

dipeptides was strain specific and *L. reuteri* LTH5448 produced significantly higher amount of γ-Glu-Ile and γ-Glu-Glu than *L. reuteri* 100-23 (Figure 3-3). This result confirmed strain specificity on synthesis of γ-glutamyl dipeptides and exclude the effect of proteolysis and rye malt.

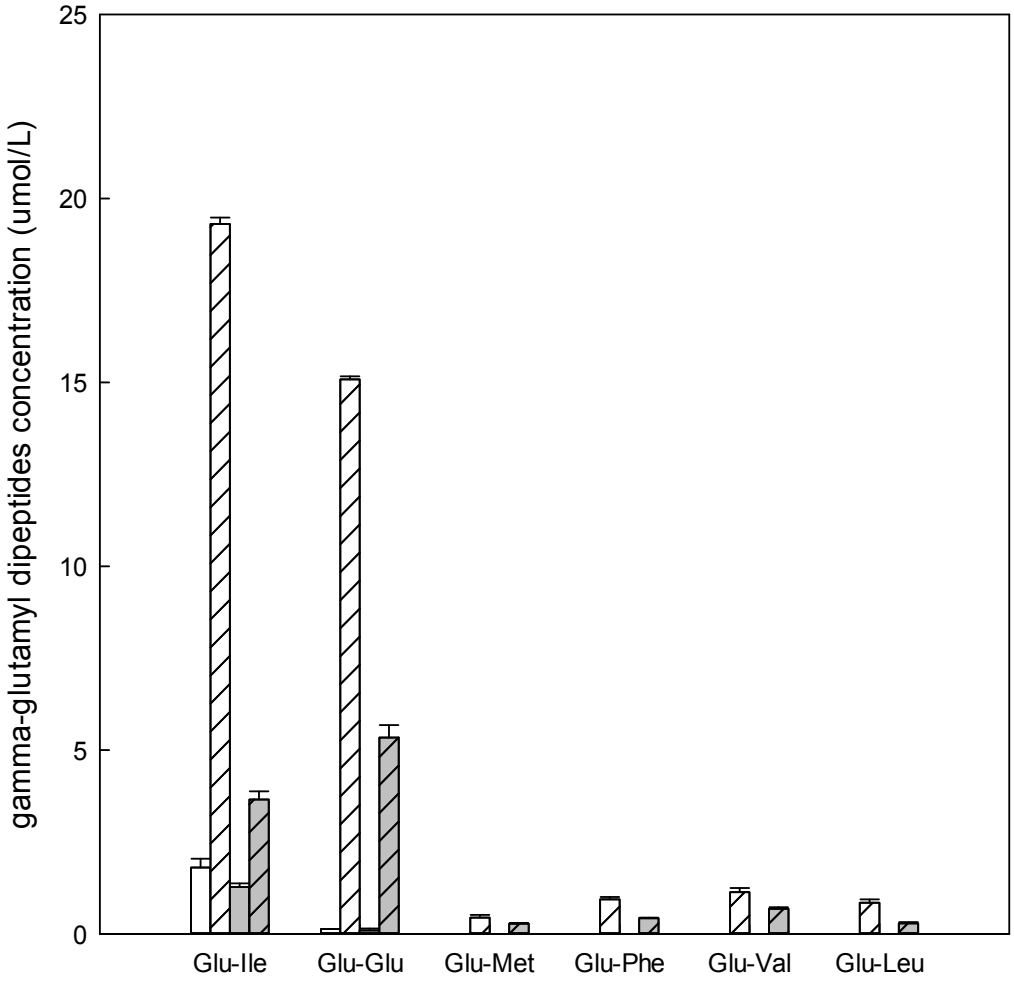

Figure 3-3 Concentration of γ-glutamyl peptides in amino acid model fermented by *L. reuteri* LTH5448 (white bar) and *L. reuteri* 100-23 (grey bar) for 0 h (non hatched bar) and 24 h (hatched bar) respectively.

3.3.6 Effect of *L. reuteri* 100-23 and *L. reuteri* LTH5448 on the salty taste

To determine whether kokumi-active γ-glutamyl peptides influence the taste of bread, a sensory evaluation was performed on sourdough bread fermented with different strains of *L. reuteri*. Sourdough fermented with *L. reuteri* LTH5448 and *L. reuteri* 100-23 had food-grade fungal protease (Flavourzyme) added to achieve the same level of proteolysis. Addition of 5% sourdough to bread was calculated to result in 5 μmol/kg of all the 6 identified γ-glutamyl dipeptides. The combined concentrations of all 6 γ-glutamyl dipeptides matched or exceeded the kokumi taste threshold (Toelstede et al., 2009; Toelstede & Hofmann, 2009). At 1.5% salt, there was no significant difference in paired comparison of the salty and preference test (Table 3-6). At 2% salt level, there is significant difference in the paired comparison of salty taste (Table 3-6).

Table 3-6 Paired comparison of salty taste and preference for sourdough bread *L.reuteri* LTH5448 and 100-23 at 2% (n=30) and 1.5% salt level (n=42).

	2% salt	**1.5%salt**
Salty taste	Significant difference	Not different
Preference	Not different	Not different

The similar results were confirmed by Just About Right test (JAR) at 2% salt level sourdough bread. About 40% panelists chose *L. reuteri* 100-23 at “very salty” or “too much salty” whereas only 27% of the panelists chose *L. reuteri* LTH5448 in the same category. Differently, about 20% panelists chose *L. reuteri* LTH5448 as “little salty” or “not salty at all” but less than 10% panelists think of *L. reuteri* 100-23 in the same category. Both of the two breads had around 50% just about right of salty taste (Figure 3-4). The similar

results were found in after taste. The sensory evaluation confirmed the significant difference of salty taste between *L. reuteri* LTH5448 and 100-23 bread.

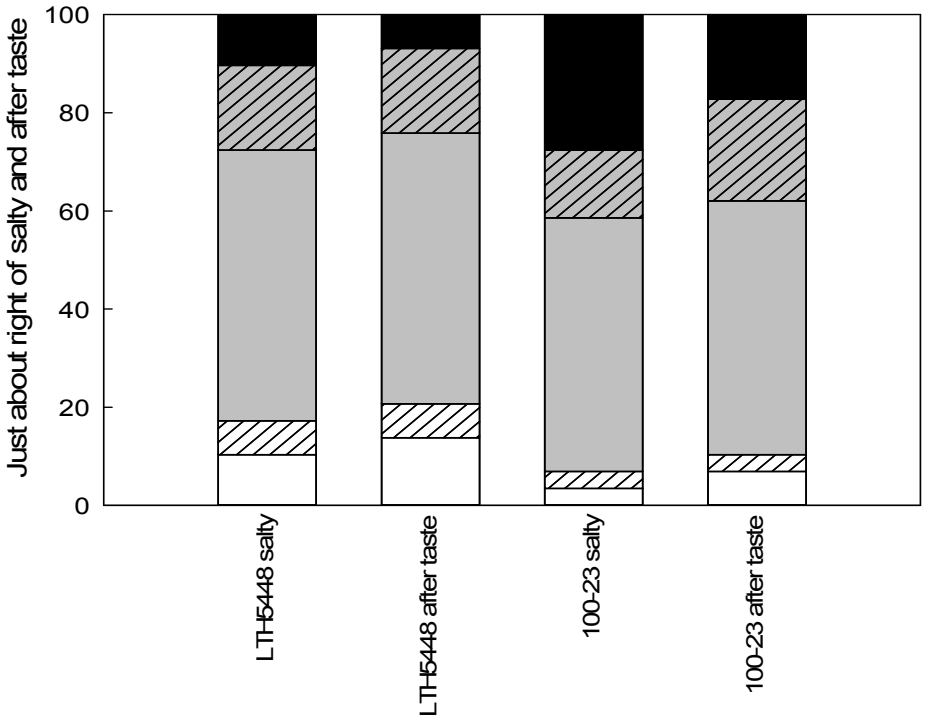

Figure 3-4 Just about right of salty and after taste of sourdough bread fermented with *L.reuteri* LTH5448 and *L.reuteri* 100-23 at 2% salt level. Bar colors indicate % of answers indicating "not salty at all" (white bars), "little salty" (white hatched bars); "just about right" (gray bars); "very salty" (gray hatched bars); and "too much salty" (black bars).

3.4 Discussion

3.4.1 Generation of γ-glutamyl peptides by *Lactobacillus*

The results of this study demonstrate the presence of γ-glutamyl dipeptides in sourdough and their formation by bacterial activity. The presence of γ-glutamyl peptides might relate to glutathione degradation or the action of microbial enzymes, such as γ-glutamyl

transferase (EC.2.3.2.2) and γ-Glu-Cys synthetase (γ-GCS) (EC.6.3.2.2), which play an important role in glutathione synthesis (Roudotalgaron et al., 1994). γ-Glutamyl transferase was found in *Penicilliums* in cheese, *Aspergillus* and *Bacillus* from soy sauce. γ-GCS produces γ-Glu-Cys from free glutamine and cysteine, which can be a substrate of glutathione synthetase (GS) to produce glutathione. Glutathione can be a substrate of γ-glutamyl transferase to generate large varieties of γ-glutamyl dipeptides (Roudotalgaron et al., 1994). The first finding of γ-glutamyl dipeptides was also attributed to the activity of γ-Glu-Cys synthetase (EC.6.3.2.2). Several LAB produce glutathione, such as *Lactococcus lactis, Streptococcus thermophilus*, and *Lactobacillus helveticus* (Roudotalgaron et al., 1994). In addition to the enzymes from *Penicilliums* or *Aspergillus*, γ-GCS from *Streptococcus agalactiae* synthesized several kinds of γ-glutamyl tripeptides (Kino et al., 2007). Interestingly, the γ-GCS from *Streptococcus agalactiae* had different substrate specificities from those of *E.coli*. The gene coding for γ-Glu-Cys synthetase (EC.6.3.2.2) was found in *L. reuteri* LTH5448 and *L. reuteri* 100-23, but not in *L. sanfranciscensis* and *L. sakei*. An amino acid model on *L. helveticus* and *L. rhamnosus* indicated that γ-glutamyl dipeptides were synthesized only in lysed cells but not in live cells (Sgarbi et al., 2013). Sgarbi et al (2013) indicated that the enzymes are in the cytoplasm and active only after cell lysis. The GGT activity is not found in *L. helveticus* and *L. rhamnosus*. Sgarbi et al (2013) indicated the bacterial enzymatic activity, which is different from GGT, might contribute to the γ-glutamyl synthesis. In this study, the gene of γ-glutamyl transferase (EC.2.3.2.2) was not found in any lactobacilli mentioned above (Accession number of *L. reuteri* LTH5448 NZ_JOOG00000000.1).

The sourdough fermented with different ingredients demonstrated that proteolysis and extended fermentation time are important factors contributing to the γ-glutamyl peptide synthesis (Table 3-3 & Table 3-4). Free amino acids or small peptides released from primary proteolysis can be the substrate for the following enzyme reaction to produce γ-glutamyl peptides. There is no significant difference between the level of γ-glutamyl dipeptides of *L. reuteri* 100-23 and *L. reuteri* 100-23 ΔgadB. γ-Glu-Glu and γ-Glu-Ile are the predominant peptides, which might relate to the high amount of Glu, Ile, Leu in cereal proteins and reflect that the synthesis have different preference on amino acids (Tkachuk & Irvine., 1969). This finding differs from the amino acid model fermented by *P. roquefortii* from cheese that homotranspeptidation product (γ-Glu-Gln) is the predominant product (Toelstede & Hofmann, 2009). The acidic and basic amino acids, such as His or Glu are poor acceptor amino acids, whereas neutral amino acids such as Met are the most preferred acceptor (Toelstede & Hofmann, 2009). However, depending on the source of GGT, the reaction show different preference such as GGT from *E.coli* prefer basic amino acids whereas mammalian GGT showed high affinities for neutral amino acids (Suzuki et al., 1986). The similar results were found on γ-GCS that the substrate specificity depends on the source of GCS (Kino et al., 2007).

During fermentation, the additional MTG not contribute to the synthesis of γ-glutamyl peptides (Appendix B Table B-2). One possible explanation is that during the sourdough fermentation, low pH (pH 3.5) is not ideal for MTG reaction (Speranza & Morelli, 2012). On the other hand, the MTG can cross-link the side chains of proteins but not result in the formation of γ-glutamyl dipeptides.

3.4.2 Relationship of γ-glutamyl peptides to taste

The results in Chapter 2 indicated that there is no significant difference between *L. reuteri* LTH5448 and *L. reuteri* 100-23 due to the comparable level of glutamate. However, in this study, salty taste between *L. reuteri* LTH5448 and *L. reuteri* 100-23 at 2% salt level were significantly different. It confirmed that except for the glutamate, γ-glutamyl dipeptides may also influence the taste (Table 3-6). Besides, compared with type I sourdough fermented by *L. sakei* and regular bread without addition of sourdough, it is obvious to see the sourdough with γ-glutamyl dipeptides significantly increase the salty taste (Table 3-2). Even though, the level of γ-glutamyl dipeptides in the bread is below the individual threshold (Table 3-1), the sum of all the kokumi active peptides in bread is still comparable to the average kokumi threshold of γ-glutamyl peptides (Roudotalgaron et al., 1994; Sforza et al., 2006; Toelstede & Hofmann, 2009). Therefore, the effect of kokumi peptides not be neglected. Free amino acids, amino acid derivatives, and small peptides enhance other tastes at subthreshold concentration. Phe and Tyr increase the intensity of umami taste at subthreshold level (Lioe et al., 2004; Frerot & Chen, 2013). Dunkel et al. (2007) mentioned the recognition threshold of γ-glutamyl dipeptides in savory base such as chicken broth or with NaCl, or glutamate was significantly lower than that in water. The kokumi threshold of glutamyl peptides in cheese is about 100-1000 lower than that in water (Dunkel et al., 2007; Toelstede et al., 2009). This indicated that the taste-taste interaction could affect the threshold and also confirm the function of taste enhancers. Also, due to the limited γ-glutamyl dipeptides quantified in this study, there may be kokumi compounds that were not accounted for.

3.5 Conclusion

In conclusion, 6 γ-glutamyl dipeptides were quantified in sourdough by LC-MS/MS. The sensory evaluation results indicated that the γ-glutamyl dipeptides may affect the taste of bread. The proteolysis and strain specificity are important in the synthesis of γ-glutamyl dipeptides. Further study is needed to investigate the enzyme activity and metabolism pathway in bacteria contributed to the kokumi peptide synthesis.

4 Fate of ACE-inhibitory peptides during the bread-making process: quantification of peptides in sourdough, bread crumb, steamed bread and soda crackers

4.1 Introduction

Certain dietary peptides inhibit the angiotensin I converting enzyme (ACE) *in vivo* (Kilara & Panyam, 2003; Korhonen & Pihlanto, 2003). Di- and tripeptides resist hydrolysis by brush border peptidases and reach the bloodstream (Iwai et al., 2005). Purified peptides or the dairy products Calpis® and Evolus® containing Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) (single letter code used to denote amino acids) moderately but consistently reduce the systolic blood pressure in hypertensive patients (Ricci et al., 2010). Knowledge on structure-function relationships of ACE-inhibitory peptides facilitates their identification *in silico*. Tripeptides with branched chain aliphatic residues at the N-terminus and proline or aromatic amino acids at the C-terminus typically display ACE-inhibitory activity (Hayes et al., 2007; Meisel, 2005; Wu et al., 2006). Hydrophobic amino acids are also preferred for the penultimate position of ACE-inhibitory tripeptides but peptides with glutamine or glutamate in the penultimate position are also ACE inhibitory (Anthony et al., 2012). For example, the IC_{50} of LQP, LIP, LLP, IPP, LPP, VPP and ILP are, 2, 2.5, 57, 5, 9.6, 9 and 83 μmol L^{-1} respectively (Miyoshi et al., 1991; Wu et al., 2006).

Proline and hydrophobic amino acids are abundant in prolamins of rye, wheat, and barley (Wieser & Koehler, 2008). ACE inhibitory peptides are frequently encrypted in the primary sequence of prolamins (Hu et al., 2011; Loponen, 2004). Proteolysis during sourdough fermentation yields peptides as major products (Gänzle et al., 2008), and

releases ACE inhibitory peptides (Hu et al., 2011; Rizzello et al., 2008). The release of ACE-inhibitory peptides was attributed to the combined activity of cereal, fungal, and microbial proteases. Particularly tripeptides with the sequence motif XPP resisted degradation and accumulated to high concentrations (Hu et al., 2011).

Sourdough has been used as a leavening agent for bread production (type I sourdoughs). The current use of sourdough additionally includes its application as a baking improver (type II sourdoughs) to improve bread quality (Brandt, 2007; Gänzle et al., 2007). Sourdoughs are also used in the production of soda crackers and steamed bread (Sugihara, 1978; Schuholz, 1989; Kim et al., 2009). Steamed bread (*mantou*) is a widely consumed breakfast item in China. Comparable products, *Dampfnudel* ("steamed noodle") and *Germknödel* ("steamed dumpling"), are produced at household level in Germany and Austria, respectively. Although the presence of ACE inhibitory peptides in sourdough was reported (Rizzello et al., 2008; Hu et al., 2011), data on their concentration in bread, steamed bread, or crackers are lacking, and the effect of the dough preparation and baking on their concentration is unknown. Peptides are subject to thermal degradation during bread baking (Buhlert et al., 2006). In bread, thermal reactions in the crust and crumb differ due to the differences in the time-temperature regime and the water activity. Soda crackers are baked at high temperatures, resembling the thermal history of the bread crust. Steamed bread is cooked with steam at 100°C, resembling the thermal history of the bread crumb.

This study aimed to determine the concentration of the ACE-inhibitory peptides, IPP, LPP, VPP, LQP, LIP, and LLP throughout the sourdough bread making process. Based on the structure-function relationships of ACE-inhibitory peptides, peptides with isoleucine or leucine at the N-terminus have equivalent ACE-inhibitory activity (see above) and IQP

and IIP thus also exhibit activity. Because these two peptides are also frequently encoded in the primary sequence of prolamins, they were additionally quantified. LC-MS/MS in multiple reaction monitoring mode was used to quantify the concentration of ACE-inhibitory peptides as described previously (Hu et al., 2011). Sourdough was fermented for use in baking corresponding to the current industrial use of type I or type II sourdoughs. To assess the effect of thermal processing on tripeptide levels, sourdoughs were used in the production of bread, steamed bread, and soda crackers.

4.2 Materials and methods

4.2.1 Strains and growth conditions

Lactobacillus reuteri TMW1.106 was grown in modified de Man, Rogosa and Sharpe medium (mMRS) (Stromeck et al., 2011) at 37°C. Agar plates were incubated under modified atmosphere (4% O_2, 20% CO_2, and 76% N_2). Inocula for sourdough fermentation were prepared by harvesting of cells from overnight cultures in mMRS at 4000×*g* for 5 min. Cells were washed twice in sterile tap water and resuspended in tap water to the initial culture volume to obtain the inoculum for dough fermentation.

4.2.2 Dough fermentation.

Rye malt flour was obtained from Laihian Mallas (Laihia, Finland), wheat flour was obtained in a local supermarket, and wheat gluten was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rye-malt gluten sourdough was prepared as previously described (Stromeck et al., 2011). In brief, 40 g of rye-malt flour was mixed with 40 g of vital wheat gluten, 240 g of tap water, 80 mL of washed culture, and 2.31 μL g^{-1} fungal protease from *Aspergillus oryzae* (Sigma-Aldrich, USA). Rye-malt sourdoughs were fermented for 96 h

at 37°C as previously described (Stomeck et al., 2011). Wheat sourdough was prepared by mixing 40 g rye malt flour, 40 g whole wheat flour, 80 mL washed culture and 40 g tap water, followed by fermentation for 24 h at 37°C. Chemically acidified doughs without addition of cultures served as a control. Controls were acidified to pH 3.66 ± 0.23 by addition of 0.27% of a mixture of acetic acid (100% w/w) and lactic acid (85% w/v) in a volumetric ratio of 1 to 4. Samples were taken after 96 or 24 h of fermentation and the pH was determined with a glass electrode. Cell counts were analyzed by plating appropriate dilutions of dough on mMRS agar. Sourdough was freeze dried and stored at room temperature for the subsequent analysis of free amino nitrogen and tripeptides, and for use in baking experiments.

4.2.3 Preparation of bread, steamed bread, and soda crackers

Bread dough was prepared using the dough formula shown in Table 4-1 with the addition of rye-malt sourdough or wheat sourdough. Ingredients were mixed in a spiral kneader (Kitchen Aid K45SS, Hobart Co. Troy, OH, USA) for 3 min. The dough was shaped after a dough rest of 30 min and proofed for 90 min at 30°C and 85% humidity in a proofer (Res-Cor, Crescent Metal Products Inc, Cleveland, OH, USA). Bread was baked in a multi-deck oven (Bakers Pride, Lachine, QC, Canada) set at a temperature of 210°C for 50 min. Steamed bread was prepared by placing the dough in a steamer (T-FAL vita cuisine, Scarborough, ON, Canada) for 40 min. To prepare soda crackers, dough was sheeted by roller after proofing and cut by a 10 inch round cutter. The weight of the dough pieces was 5 ± 0.5 g. Soda crackers were baked at 210°C for 15 min. Bread was also prepared in a straight dough process (Table 4-1) to quantify tripeptides in bread prepared without sourdough. The dough pH was adjusted to pH 4.5, 5.0, or 5.5 with a mixture of

acetic and lactic acids (ratio 1:4, v/v). Kneading, proofing, and baking were carried out as described above. Samples for peptide analysis were taken after kneading of the dough, after proofing of the dough, and from the bread crumb, steamed bread or crackers. Samples were freeze dried and ground into a powder for further analysis. Samples were extracted and analyzed in triplicate technical repeats.

Table 4-1 Recipes for preparation of bread, soda crackers, and steamed bread.

Ingredients (g)	3% rye-malt sourdough	6% rye-malt sourdough	10% wheat sourdough	20% wheat sourdough	Straight dough
wheat flour	97	94	90	80	100
rye malt sourdough[a)]	3	6	-	-	-
wheat sourdough [a)]	-	-	10	20	-
Yeast	2	2	2	2	2
Sugar	2	2	2	2	2
Salt	2	2	2	2	2
Water	60	60	60	60	60

[a)] Sourdough was replaced by an equivalent amount of chemically acidified dough as control.

4.2.4 Maillard reaction model

A Maillard reaction model system (Lancker et al., 2010) was used with modifications to obtain a pH value resembling the pH of sourdough bread (5.0). In brief, IQP, LQP, IIP, LIP, LLP, IPP, LPP, and VPP were dissolved at a concentration of 0.25 µmol L^{-1} each in acetate buffer (50 mmol L^{-1} sodium acetate, 20 mmol L^{-1} acetic acid, pH 5.0) containing 1 mmol L^{-1} lysine and 1 mmol L^{-1} glucose, respectively. The samples were heated at 100°C for 5 min or 20 min in an incubator and immediately cooled in an ice bath afterwards.

Subsequently, they were diluted with aqueous acidified methanol (30% v/v methanol, 0.1% formic acid) at a ratio of 1:10, stored at -20 °C, and analyzed by LC-MS/MS.

4.2.5 Quantification of tripeptides by LC-MS/MS analysis

The extraction and quantification of peptides were carried out as described by Hu et al. (2011) using a 1200 series HPLC unit (Agilent Technologies, Palo Alto, CA, USA) coupled to a 4000 QTRAP LC-MS/MS System (MDS SCIEX-Applied Biosystems, Streetsville, ON, Canada). Peptides were separated on a Luna C18 RP-HPLC column (5 µm, 250 x 4.6 mm, Phenomenex, Torrance, CA, USA) and eluted at 0.5 mL min^{-1} and 40°C in 0.1% formic acid in water (Eluent A) and 0.1% formic acid in acetonitrile (Eluent B) with the following gradient: 4 min, 0% B; 16 min, 7% B; 44 min, 0% B; 51.5 min, 45% B; followed by re-equilibration to 0% B for 10 min. Analytes were quantified using multiple reaction monitoring mode (MRM). Lyophilized sourdoughs were extracted with 30% (v/v) methanol in 0.1% aqueous formic acid, solids were removed by centrifugation at 10000 x g for 10 min. Samples were stored at -20°C until analysis.. External standards (IQP, LQP, IIP, LIP, LLP, IPP, LPP and VPP) were purchased from United Peptide Corporation (Bethesda, MD, USA). LC-MS/MS parameters for the 8 tripeptides were selected as follows: IQP: Transition 357→242 (*m/z);* declustering potential DP, 31; collision energy CE 19(V); collision exit potential CXP 6, retention time (min), 28.9. The corresponding parameters for the other peptides were: LQP:357→242 *m/z;* 31; 19 V; 6; 29.6 min; IIP: 342→116 *m/z*; 36; 19 V; 8; 34.6 min; LIP: 342→116 *m/z*; 36; 19 V; 8; 35.8 min; LLP: 342→116 *m/z*; 36; 19 V; 8; 36.7 min; IPP: Transition 326→213 (*m/z*); 36; 25V; 12; 28.8 min; LPP: 326→213 *m/z*; 36; 25 V; 12; 29.6 min; VPP: 326→213 *m/z*; 31; 25 V; 12; 24.0 min. Calibration curves consisted of seven concentrations ranging from 0.001 to 1 mg L^{-1}.

4.2.6 Statistical and data analysis

Triplicate independent sourdough fermentation and baking experiments were carried out unless otherwise stated. Statistical analysis of peptide yield was performed by analysis of variance (ANOVA) with the procedure PROC GLM using the Statistical Analysis System (SAS) V.9.2 (SAS Institute Inc., Cary, NC, USA). Values for peptide concentrations or peptide yields were considered significantly different at a 5% error level ($P < 0.05$).

4.3 Results

4.3.1 Quantification of tripeptides by LC-MS/MS

Liquid chromatography coupled with mass spectrometry in MRM mode was used for the selective and sensitive quantification of peptides. A previously established method (Hu et al., 2011) was modified to additionally include IQP, IIP, LIP and LPP, peptides with known or predicted ACE-inhibitory activity. The calibration curves for the eight tripeptides were linear in the concentration range from 0.005 to 1 mg L^{-1}. The molecular masses of LQP and IQP are identical but the peptides could be distinguished by their retention times. Likewise, IPP and LPP as well as IIP, LIP, and LLP were identified by their retention times even though they have identical mass spectra. Isobaric peptides with isoleucine (IPP and IQP) eluted earlier than the corresponding peptides with leucine (LPP and LQP, respectively), reflecting the higher hydrophobicity of leucine. Accordingly, the elution order of IIP, LIP, and LLP reflected the hydrophobicity of leucine.

4.3.2 Fermentation parameters

Wheat doughs were fermented with starter cultures, with or without fungal protease. The cell counts of wheat sourdoughs and rye-malt gluten sourdoughs were 5×10^9 and 4.5×10^8 cfu g^{-1} after 24 and 96 h of fermentation, respectively. Cell counts of chemically acidified doughs were below the detection limit of 10^5 cfu g^{-1} throughout fermentation, excluding a contribution of microbial metabolism to peptide concentrations. Wheat sourdoughs and rye-malt gluten sourdoughs were acidified to pH 3.5 ± 0.0 and 3.7 ± 0.0 after 24 and 96 h of fermentation, respectively. The pH values of chemically acidified wheat dough and chemically acidified rye-malt dough were 4.0 ± 0.0 and 3.7 ± 0.0, respectively, throughout the incubation time.

4.3.3 Effect of yeast, flour and pH on tripeptide concentrations

The formation of ACE-inhibitory peptides in sourdough fermentation was previously reported but their concentration in straight doughs fermented only with baker's yeast is unknown. To assess the formation of peptides in a straight dough process, bread was produced without sourdough (Table 4-1) after acidification to pH 4.5, 5.0, or 5.5. Experiments were carried out in duplicate and tripeptides were quantified after kneading, proofing and baking. The concentrations of IQP, LIP, IPP, LPP andVPP were 100 fold lower than those in sourdough (Table 4-2); LQP, IIP and LLP were below the limit of detection. The contribution of yeast or cereal enzymes to tripeptide accumulation in straight dough processes was thus negligible.

Table 4-2 Concentration and yield of tripeptides in rye malt-gluten sourdough, and tripeptide yield in bread dough kneading, after proofing, and in bread crumb after baking. Rye malt-gluten sourdough was added at a level of 3% or 6%. Chemically acidified rye malt-gluten dough was used as control. Data are shown as means ± standard deviations of triplicate independent fermentations analysed in quadruplicate

	IQP	LQP	IIP	LIP	LLP	IPP	LPP	VPP
	Peptide concentration in rye-malt gluten sourdough							
[µmol kg^{-1}]	174 ± 9	255±11	24.9±3.6	303±26	12.3±1.7	473±44	365±31	47.3±4.2
yield [%][a)]	**Peptide yield in bread dough and bread with 3% rye-malt sourdough**							
kneading	203±12^{a}	50±4^{e}	78±3^{d}	20±1^{f}	17±6^{f}	115±6.5^{c}	135±5^{b}	138±5^{b}
proofing	189±34^{a}	39±6^{e}	63±4^{e}	14±2^{f}	19±4^{f}	122±15^{c}	148±9^{b}	157±11^{b}
crumb	161±32^{b}	41±10^{e}	53±8^{e}	16±3^{f}	15±3^{f}	b±5^{e}	53±10^{e}	102±32^{d}
	Peptide yield in bread dough and bread crumb with 6% rye-malt sourdough							
kneading	120±5^{a}	86±8^{b}	82±7^{b}	42±5^{c}	40±9^{c}	104±4^{a}	105±10^{a}	112±7^{a}
proofing	189±20^{a}	39±8^{c}	63±6^{b}	14±3^{c}	19±6^{c}	122±12^{a}	148±11^{a}	157±10^{a}
crumb	118±14^{a}	68±18^{b}	68±8^{b}	28±4^{c}	27±6^{c}	68±14^{b}	40±6^{b}	104±40^{b}
	Peptide concentration in rye-malt gluten control dough							
[µmol kg^{-1}]	115±10	79.3±6.6	13.5±1.5	89.4±1.8	4.4±0.3	318±29	226±32	62.7±6.1
yield [%]	**Peptide yield in bread dough and crumb with 3% rye-malt control dough**							
kneading	187±47^{a}	94±1^{b,c}	124±16^{b}	68±17^{c}	58±31^{c}	189±13^{a}	214±25^{a}	160±24^{a}
proofing	176±21^{a}	66±9^{c}	86±9^{b}	38±4^{c}	26±9^{c}	209±19^{a}	223±21^{a}	172±24^{a}
crumb	107±4^{b,c}	57±1^{c}	53±1^{c}	26±1^{c}	19±4^{c}	92±21^{b,c}	78±16^{b}	101±3^{b,c}
	Peptide yield in bread dough and crumb with 6% rye-malt control dough							
kneading	176±7^{b}	138±24^{c}	141±30^{c}	97±2^{d}	58±3^{e}	182±16^{a}	179±18^{b}	152±9^{c}
proofing	213±22^{a}	143±2^{c}	146±5^{c}	72±2^{e}	33±3^{f}	201±3^{a}	171±4^{b}	136±1^{c}
crumb	138±17^{c}	170±62^{d}	104±14^{d}	88±5^{d}	55±23^{e}	113±17^{d}	54±12^{e}	57±21^{e}

Peptide concentrations for processing and baking of the same sample differ significantly if they do not share a common superscript ($P < 0.05$).

[a)] The yield was calculated on the basis of the concentration of the peptides in sourdough and the dilution factor of the sourdough in the respective dough formula. A yield of 100% indicates that the amount of tripeptides present in the sourdough remains unchanged during further processing to bread.

4.3.4 Concentration of tripeptides in different sourdoughs, bread dough, and bread

Previous investigations reported tripeptide levels in rye malt-gluten sourdough fermented according to type II technology with extended fermentation times, but not in wheat sourdoughs fermented for 24 h (type I sourdoughs). The concentration of tripeptides in sourdough was compared to rye-malt gluten sourdoughs. Data for rye-malt gluten sourdoughs are shown in Table 4-2; data obtained for wheat sourdough are shown in Table 4-3. In rye-malt gluten sourdough, tripeptides with the highest concentration were IPP, LPP, LIP and LQP at quantities of 473, 365, 303 and 255 μmol kg^{-1} respectively (Table 4-2), exceeding the IC_{50} up to 500 times. Tripeptide concentrations in chemically acidified control dough were lower than in sourdough (Table 4-2), confirming the contribution of peptidases of lactobacilli to the release of tripeptides during fermentation (Hu et al., 2011).

Rye-malt gluten was added to bread dough at a level of 3% or 6%. To compare the effect of kneading, proofing and baking on tripeptide levels, yield was calculated relative to the concentration in the sourdough. The bread making procedure affected tripeptide levels differently. The yield of IQP, LQP, IIP, LIP and LLP changed substantially during kneading and proofing but remained relatively stable during baking. These rapid changes during kneading likely reflect peptide release or degradation owing to protease activity derived from the sourdough. Peptide yields in dough made with chemically acidified controls were substantially different from peptide yields in dough made with sourdough, again reflecting a contribution of microbial peptidases to peptide release and degradation (Table 4-2). In contrast, the concentrations of IPP, LPP, and VPP remained relatively stable during kneading and proofing of dough made with rye-malt gluten sourdough, but their yields were strongly reduced after baking.

Table 4-3 Concentration and yield of tripeptides in wheat sourdough, and tripeptide yield in bread dough after kneading, after proofing, and in bread crumb after baking. Wheat sourdough was added at a level of 10% or 20%. Chemically acidified wheat dough was used as control. Data are shown as means ± standard deviations of triplicate independent fermentations analysed in quadruplicate.

	IQP	LQP	IIP	LIP	LLP	IPP	LPP	VPP
	Peptide concentration in wheat sourdough							
[µmol kg^{-1}]	9.2±0.7	31.0±0.8	6.1±0.1	16.8±0.5	1.5±0.1	58.2±0.2	43.7±1.1	9.1±0.4
yield [%][a)]	**Peptide yield in bread dough and bread with 10% wheat sourdough**							
kneading	165±22^{a}	48±10^{c}	57±7^{c}	30±10^{c}	12±1^{d}	105±14^{b}	163±18^{a}	127±25^{b}
proofing	145±13^{a,b}	41±7^{c}	47±7^{c}	27±5^{d}	11±1^{d}	103±2^{b}	141±3^{a}	114±7^{b}
crumb	120±2^{b}	59±6^{c}	59±8^{c}	49±5^{c}	20±4^{d}	61±7^{c}	49±5^{c}	72±11^{c}
	Peptide yield in bread dough and bread crumb with 20% wheat sourdough							
kneading	139±16^{a}	94±5^{b}	99±5^{b}	93±2^{b}	59±7^{c}	99±5^{b}	110±3^{b}	114±3^{b}
proofing	115±14^{b}	83±12^{b,c}	87±8^{b}	85±13^{b,c}	47±9^{c}	93±1^{b}	94±11^{b}	99±10^{b}
crumb	82±7^{c}	67±7^{c}	68±7^{c}	67±5^{c}	52±11^{c}	54±3^{c}	38±3^{c}	57±4^{c}
	Peptide concentration in wheat control dough							
[µmol kg^{-1}]	13.8±1.0	41.2±1.1	7.3±0.2	29.3±0.9	0.6±0.1	50.7±3.1	40.5±1.5	9.0±0.2
yield [%][a]	**Peptide yield in bread dough and crumb with 10% whole wheat control dough**							
kneading	110±7^{d}	25±2^{g}	44±3^{f}	22±1^{g}	25±9^{g}	115±5^{d}	139±8^{c}	87±2^{e}
proofing	181±4^{a}	48±1^{f}	42±5^{f}	16±1^{g}	36±10^{g}	63±14^{e}	148±21^{c}	86±1^{e}
crumb	160±24^{b}	52±6^{f}	54±6^{f}	27±4^{g}	27±3^{g}	67±3^{e}	49±4^{e}	55±4^{f}
	Peptide yield in bread dough and crumb with 20%wheat control dough							
kneading	158±30^{c}	65±1^{e}	60±13^{e}	35±5^{f}	17±4^{f}	114±11^{d}	204±6^{a}	110±10^{d}
proofing	161±3^{c}	58±3^{e}	77±1^{e}	36±1^{f}	18±1^{f}	113±5^{d}	182±0^{b}	110±3^{d}
crumb	99±2^{d}	62±8^{e}	73±9^{e}	49±1^{e}	127±25^{d}	55±8^{e}	53±11^{e}	55±13^{e}

Peptide concentrations for processing and baking of the same dough differ significantly if they do not share a common superscript ($P < 0.05$).

[a)] The yield was calculated on the basis of the concentration of the peptides in sourdough and the dilution factor of the sourdough in the respective dough formula. A yield of 100% indicates that the amount of tripeptides present in the sourdough remains unchanged during further processing to bread.

Peptide concentrations in wheat sourdoughs fermented for 24 h were considerably lower when compared to the rye-malt gluten sourdough fermented for 96 h (Table 4-2 and Table 4-3). Peptide levels in sourdough and chemically acidified controls were not substantially different (Table 4-3), indicating that the short fermentation time did not allow for a substantial contribution of microbial peptidases. These lower peptide concentrations in sourdough were partially compensated for by a higher sourdough addition to bread dough. Comparable to rye-malt gluten sourdoughs, the yields of IPP, LPP, and VPP were affected by baking rather than kneading and proofing (Table 4-3).

4.3.5 Effect of thermal treatment during baking and steaming on tripeptide concentrations

To assess the influence of thermal treatment in more detail, tripeptide concentrations in steamed bread, bread crumb and soda crackers were compared (Figure 4-1). Rye-malt gluten sourdough (3%) or wheat sourdough (10%) were included in the recipes. Products were prepared from the same dough and the three products were treated separately only after kneading and proofing. This procedure ensures that changes in peptide concentration during the dough making procedure are essentially identical in all three products. In soda crackers prepared with rye-malt gluten and wheat sourdoughs, tripeptide concentrations were generally less than 0.1 and 1 μmol (kg DM)$^{-1}$, respectively. IIP and LLP concentrations were below the detection limit in all crackers; the LPP concentration was below the detection limit in soda crackers prepared with wheat sourdough (Figure 4-1). The concentrations of IQP, LQP, IIP, LIP, LLP, and VPP in bread crumb and steamed bread were not significantly different; concentrations of IPP and LPP were significantly higher in steamed bread when compared to bread crumb.

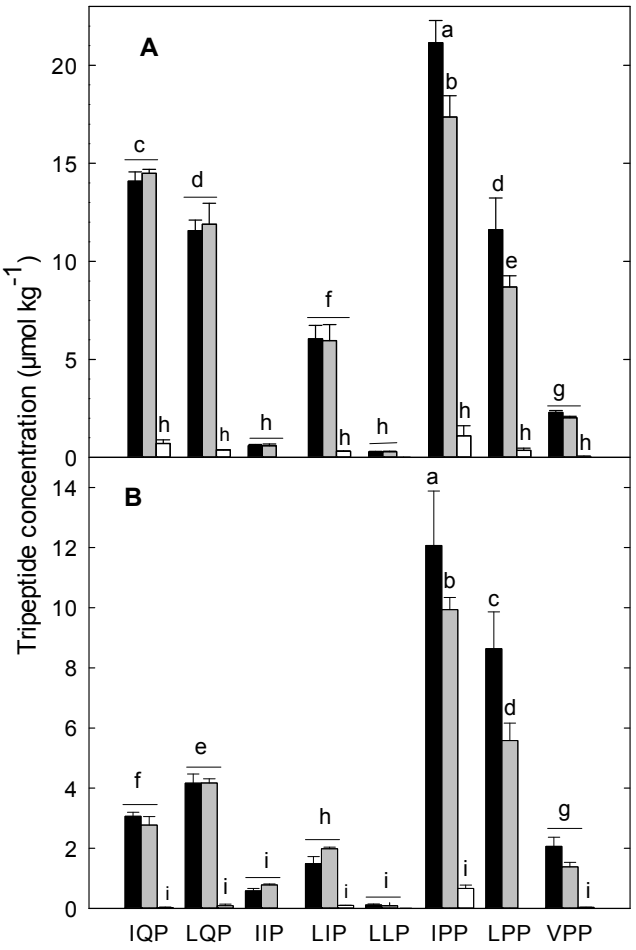

Figure 4-1 Tripeptide concentration of steamed bread (black bars), bread crumb (grey bars), and soda crackers (white bars). (Results are shown as means ± standard deviation of triplicate independent experiments analysed in triplicate.) **Panel A**: Dough prepared with addition of 3% rye-malt sourdough. **Panel B**: Dough prepared with addition of 10% wheat sourdough. The different products were prepared from the same dough to minimize experimental error owing to generation of elimination of tripeptides during kneading and proofing. (Data are shown as means ± standard deviations of triplicate independent experiments analyzed in triplicate.) Values in the same panel with different superscript differ significantly ($P < 0.05$).

4.3.6 Behavior of tripeptides in a Maillard reaction model

Baking but not steaming exerted a strong and sequence-specific effect on the concentrations of ACE-inhibitory peptides. To understand the different effects of baking or steaming on tripeptide concentrations, a Maillard reaction model was used at 100°C and pH 5.0 to monitor the concentration of tripeptides after exposure to glucose in the presence of lysine (Table 4-4). With the exception of IIP, the concentrations of all tripeptides decreased to 30 – 80% of their initial levels after 20 min of incubation (Table 4-4). LPP was the most sensitive to degradation, followed by LIP and VPP. Despite differences between the Maillard reaction model and the concentration of reactants during baking, these results indicate that the primary sequence of peptides influences their reactivity.

Table 4-4 Degradation of tripeptides in a Maillard reaction model. The initial concentration of each peptide was 0.25 μmol L^{-1}; shown is the percentage of peptide remaining after 5 and 20 min of heating at 100°C. Data are shown as means ± standard deviation of triplicate independent experiments.

	IQP	LQP	IIP	LIP	LLP	IPP	LPP	VPP
5 min	71±2	74±1	100±2	63±2	83±2	97±4	53±1	77±3
20 min	60±2	69±2	100±2	54±2	79±1	67±1	31±2	58±3

4.4 Discussion

To date, the development of food products enriched with ACE-inhibitory peptides has focused on dairy products (Kilara & Panyam, 2003; Seppo et al., 2003). Cereal products are highly suitable for the release of ACE-inhibitory peptides by lactic fermentations but have not yet been considered (Hu et al., 2011; Loponen, 2004; Rizzello et al., 2008). Different from dairy products, cereal products generally undergo heat treatment after fermentation. Previous reports demonstrated that ACE-inhibitory peptides accumulate in

sourdough to concentrations exceeding their *in vivo* active levels about 100 fold (Hu et al., 2011) but their concentration in final products was not determined. This study established the effect of sourdough fermentation, dough preparation and baking or steaming on bioactive peptides. To our knowledge, this is the first report on ACE-inhibitory peptides in baked goods. Bread crumb and steamed bread but not soda crackers could be enriched with ACE-inhibitory peptides.

Concentrations of ACE-inhibitory peptides in bread prepared using a straight dough process were 10-fold lower than their concentrations in sourdough bread, conforming to the low proteolytic activity in straight dough processes (Thiele et al., 2002; Gänzle et al., 2008). This study considered long-time fermented rye-malt gluten sourdoughs (type II sourdoughs) (Hu et al., 2011) as well as rye-malt wheat sourdoughs fermented for 24 h (type I sourdoughs). The latter sourdoughs exhibited substantially lower tripeptide levels. However, type I sourdoughs are added to bread dough at a higher level and the lower peptide concentration is partially compensated for by the higher dosage (Brandt, 2007).

At the dough stage, the concentration of peptides may be altered by yeast metabolism as well as protease activity. This study used lyophilized sourdoughs, maintaining the enzyme activities. Accordingly, peptide concentrations deviated from the theoretical yield already after kneading, approximately 5 min after the addition of water to flour. The yields of IQP, IPP, LPP, and VPP were generally over 100% after kneading, indicating that enzymatic release and degradation resulted in a net release of peptides. In contrast, yields of LQP, IIP, LIP and LLP were generally below 100% after kneading, corresponding to a net degradation of the peptides. These differences reflect the different sensitivity of peptides to proteolytic degradation. Proline-containing peptides including LLP and LQP

are degraded by proteases in rye malt as well as by peptidases of lactobacilli (Hu et al., 2011; Loponen et al., 2009; Mizuno et al., 2004), whereas tripeptides with two proline residues at the C-terminus (XPP) are highly resistant to proteolysis (Mizuno et al., 2004; Hu et al., 2011). The contribution of peptidases of lactobacilli to the release and degradation of tripeptides (Hu et al., 2011) was confirmed by comparison to chemically acidified doughs. Long-term fermentation of rye-malt sourdoughs results in lysis of bacterial cells and release of intracellular peptidases (Gänzle et al., 2008; Stromeck et al., 2011). With relatively few exceptions, peptide concentrations were not altered during proofing. This supports the notion that yeasts do not contribute to proteolytic activity and that their metabolism reduces amino acid but not peptide concentrations (Thiele et al., 2002; Gänzle et al., 2008).

During baking, peptide concentrations are altered by enzyme activity until the crumb reaches the denaturation temperature of proteases, and by thermal reactions. Two lines of evidence support the interpretation that changes after baking are mainly attributable to chemical reactions: (i) Peptides that showed the largest decrease during baking, i.e. IPP, LPP and VPP, were not susceptible to proteolytic degradation during sourdough fermentation (Hu et al., 2011) or during kneading and proofing (Table 4-2 & Table 4-3). (ii) Peptides that showed the largest decrease during baking – peptides with the sequence XPP - were also susceptible to degradation in the Maillard reaction model. Past studies suggest that di- and tripeptides are relatively stable up to a temperature of 100°C (Lan et al., 2010; Van Lancker, Adams, & De Kimpe, 2011). However, thermal degradation of peptides is dependent on the amino acid sequence (Fernandez-Garcia et al., 2003). Peptides

are rapidly modified by thermal reactions at temperatures exceeding 100°C (Lan et al., 2010).

The peptide levels in products with a different thermal history during baking or steaming reflect the sensitivity of peptides to thermal degradation. During baking of soda crackers, water evaporates and the product temperature increases to levels between 100°C and the over temperature of 210°C. Tripeptide levels in soda crackers were more than 10-fold lower when compared to the dough before baking, or bread. Bread crumb and steamed bread remain hydrated and the temperature reaches only 100°C. With the noticeable exception of XPP peptides, peptides in bread crumb were generally not reduced during baking, and peptide concentrations were not different between bread crumb and steamed bread. Differences in the concentrations of IPP and LPP in bread crumb and steamed bread may relate to the different rates of heating.

The tripeptide concentrations in bread and the amount of tripeptides in 100 g of bread are compared to the *in vitro* active concentrations in Table 4-5. The concentrations of 5 peptides are equivalent to or higher than their *in vitro* IC_{50}. Clinical trials with high blood pressure patients indicate that consumption of 3–6 mg ACE-inhibitory peptides per day reduces systolic blood pressure (Mizuno et al., 2005; Ricci et al., 2010). Although cereal products produced in this study were not evaluated *in vivo,* an initial assessment of their bioactivity can be made on the basis of the comparison of the peptide concentrations with consumption levels of the same peptides with demonstrated *in vivo* activity. One serving of bread prepared with 6% rye-malt gluten sourdough (100 g) contains 3.4 mg ACE-inhibitory peptides, an amount within the range known to be active *in vivo*. Moreover, fermented cereals and specifically wheat sourdough contain ACE-inhibitory peptides in

addition to those quantified in this study and can be additionally enriched with γ-aminobutyrate (Rizzello et al., 2008; Stromeck et al., 2011). Bread supplemented with rye malt sourdough thus likely provides *in vivo* active levels.

Table 4-5 Comparison of the concentration of ACE-inhibitory peptides present in bread produced with the addition of 6% rye-malt gluten sourdough to the concentration or the amount that are active *in vitro* or *in vivo* (Myoshi et al., 1991; Wu et al., 2006; Mizuno et al., 2005; Ricci et al., 2010).

	Tripeptide concentration (μmol kg^{-1})	IC_{50} (μmol L^{-1})	Amount of tripeptides (mg) in one serving (100 g) of bread
IQP	10	NA[a)]	0.4
LQP	27	2	1.1
IIP	3	NA	0.1
LIP	13	2.5	0.5
LLP	0	57	0
IPP	8	9	0.3
LPP	22	9.6	0.8
VPP	5	5	0.2

[a)] NA, IC_{50} data for ACE inhibitory activity are not available.

4.5 Conclusion

The bread-making steps, including kneading, proofing and baking affect the stability of peptides. The stability of determined peptides related to the amino acid composition and sequence. The X-PP was the most stable peptides during dough stage but decreased during baking whereas other ACE tripeptides remained stable during baking but decreased during the dough stage. The cumulative concentrations of 8 ACE-inhibitory peptides in steamed bread and baked bread likely meet *in vivo* active concentrations.

5 Conclusions and future directions

5.1 General conclusions

Results of the studies undertaken in this PhD research indicated that the generation of bioactive and taste active amino acids and peptides during sourdough fermentation. In addition, sensory properties of bread with sourdough fermented with *L. reuteri* accumulating glutamate or γ-aminobutyrate (GABA) were investigated. Sourdough was fermented with the GABA producing *L. reuteri* 100-23 and LTH5448 as well as the glutamate accumulating *L. reuteri* 100-23 ΔgadB and TMW1.106. A consumer panel detected significant differences in the taste of bread with the addition of 6% sourdough fermented with glutamate or GABA producing *L. reuteri*. Remarkably, this difference was also detected when GABA producing *L. reuteri* 100-23 was compared to its glutamate-producing isogenic mutant *L. reuteri* 100-23ΔgadB. The intensity of the salty taste of sourdough bread produced with 1% (flour base) salt was equivalent to that of the reference bread produced with 1.5% salt. A trained panel found that sourdough bread (1% or 2% NaCl flour base) had a higher intensity of sour and umami tastes when compared to the reference bread with the same salt content. Bread produced with sourdough fermented with *L. reuteri* 100-23ΔgadB consistently had a higher umami taste intensity when compared to the other sourdough bread. Neither sourdough addition nor NaCl concentration influenced bread volume or texture.

This work is one of few research studies that explore food fermentation from the gene to the function of compounds during fermentation as well as sensory analysis of fermented foods. The gene coding for glutamate decarboxylase (gadB) has already been previously

researched, which converts glutamate to GABA and contributes to the acid resistance of lactobacilli (Su et al., 2011). Comparison of the isogenic mutant and wildtype indicated that glutamate concentration was significantly different and sensory evaluation demonstrated that glutamate and gadB contributed to the taste of bread.

In sourdough, γ-Glu-Glu, γ-Glu-Leu, γ-Glu-Ile, γ-Glu-Phe, γ-Glu-Met, γ-Glu-Val with known taste thresholds were identified by liquid chromatography-tandem mass spectrometry in MRM mode. γ-Glutamyl dipeptides were generally found in higher concentrations when compared to the chemically acidified controls. The amino acid model demonstrated how microbial activity contributes to the synthesis of those peptides. Sourdough fermented with different ingredients at different times indicated proteolysis was important for the generation of γ-glutamyl dipeptides. Sourdoughs fermented with 4 strains of *Lactobacillus reuteri* had different concentrations of γ-Glu-Glu, γ-Glu-Leu and γ-Glu-Met, which indicated that there was a strain-specific difference in enzyme activity. Sensory evaluation also suggested that γ-glutamyl dipeptides increased the salty taste in bread compared to the regular and type I sourdough bread. Also, the different salty taste between *L. reuteri* LTH5448 and *L. reuteri* 100-23 indicated that in addition to the glutamate, the peptides also affected the taste of bread.

This work also explored angiotensin-converting enzyme (ACE) inhibitory peptides in comparison to the concentration of ACE-inhibitory peptides at different stages of the bread-making process, including kneading, proofing, and final products. Steamed bread, baked bread, and soda crackers were produced with the addition of rye malt sourdoughs to assess products with different thermal treatments. Eight tripeptides with known or predicted ACE-inhibitory activities were quantified by LC/MS in multiple reaction

monitoring (MRM) mode. In wheat sourdough and rye-malt gluten sourdough, IPP was the predominant tripeptide at 58 and 473 μmol kg^{-1}, respectively, followed by LQP, IQP, and LPP. During the bread-making process, peptide concentrations were modified by the enzymatic conversions at the dough stage, and by thermal reactions during baking. The concentrations of IPP, LPP and VPP remained stable during dough preparation but decreased during thermal treatment; the concentrations of other peptides were changed at the dough stage but remained relatively stable in the final products. The cumulative concentration of 8 ACE-inhibitory peptides in steamed bread and bread crumb exceeded 60 μmol kg^{-1}, while soda crackers contained less than 3 μmol kg^{-1}. The peptide levels in bread thus likely meet *in vivo* active concentrations.

Current research suggests that high salt intake is one of the major risk factors for cardiovascular disease (Cook et al 2007; He & MacGregor 2001). The average daily salt intake in developed countries exceeds WHO recommendation by 2 fold (World Health Organization 2007). Therefore, it is important to produce sodium-reduced foods without sacrificing food quality. NaCl is an important contributor to taste. Also, NaCl determines the texture of bread since it strengthens the gluten network at the dough stage. Because of the multiple roles of NaCl in sensory and technological properties of bread, reducing NaCl in bread without compromising taste, volume and texture of bread is a challenge.

Functional culture used for bread fermentation may induce salt reduction and blood pressure control in multiple levels. Sourdough bread with accumulated glutamate and γ-glutamyl dipeptides have a much saltier taste than control bread at the same salt level. The texture and volume of bread are improved during sourdough fermentation. GABA has multiple physiological functions, including antihypertensive properties. GABA and ACE-

inhibitory peptides can be accumulated during sourdough fermentation by specific strains, including *L. reuteri* 100-23 and *L. reuteri* LTH5448 (Stromeck et al., 2011). ACE inhibitory tripeptides were accumulated during sourdough fermentation with addition of fungal protease and exceeded the IC_{50} by 100-1000 fold (Hu et al., 2011). After baking, the concentration of 8 ACE-peptides in bread crumb exceeded 60 μmol kg^{-1}, which likely meets *in vivo* active concentrations. Through the combination of glutamate, kokumi peptides, ACE-inhibitory peptides as well as GABA, a new functional bread can be produced with low sodium and bioactive compounds without adverse effects on taste or other quality attributes.

5.2 Future directions

This work found that there was a significant difference in salty taste of bread fermented with *L. reuteri* 100-23 and *L. reuteri* LTH5448. Both of these strains accumulated γ-glutamyl dipeptides. It is known that γ-Glu-X or γ-Glu-X-Gly (X can represent an amino acid or amino acid derivative other than Cys), such as γ -Glu-Gln, γ-Glu-Abu and γ-Glu-Abu-Gly, are kokumi active compounds (Nishiuchi et al., 2013). Thus, the difference between bread fermented with *L. reuteri* 100-23 and *L. reuteri* LTH5448 might be related to those undetermined compounds. Gene knock down or HPLC combined with reconstitution test can be used to investigate the contribution of other taste active compounds to the bread taste.

GGT, the known enzyme related to γ-glutamyl dipeptide synthesis, was found in cheese cultures. Other research in cheese indicated that unknown endogenous milk enzymes and the enzymes from lactobacilli might contribute to the γ-glutamyl dipeptide synthesis (Sgarbi et al., 2013). In this work, GGT activity was not found in any of the

lactobacilli. However, LC-MS/MS from the amino acid model and sourdough fermentation demonstrated that kokumi peptides were generated by lactobacilli. Further research is needed to investigate the enzyme and metabolic pathway related to the synthesis of kokumi peptides by lactobacilli. A patent on γ-Glu-Abu from yeast and yeast extract claimed that γ-Glu-Abu, another kokumi active compound, was related to enhanced γ-glutamylcysteine synthetase (γ-GCS) activity and attenuated glutathione synthetase activity (Nishiuchi et al., 2013). Another patent mentioned that γ-Glu-Abu was produced by the action of γ-GCS from glutamate and aminobutyric acid (Yamagishi & Nishiuchi, 2015). γ-GCS and glutathione synthetase (GS), depending on their original cultures, might have broad substrate specificities and synthesize several kinds of γ-glutamyl peptides (Kino et al., 2007). Knocking down the gene coding for γ-GCS in *L. reuteri* and comparing the level of γ-glutamyl dipeptides and tripeptides between wild type and mutant can demonstrate the contribution of γ-GCS to the synthesis of kokumi peptides. Sensory evaluation of bread fermented with wild type and isogenic mutant may prove the effect of γ-glutamyl peptides on the taste of bread.

A series of Amadori rearrangement products, pyroglutamic acid (pGlu) and pyroglutamyl peptides, as well as lactoyl amino acids are found in cheese, soy sauce and meat products. Those compounds impart umami taste and enhance other taste sensations in food (Ottinger & Hofmann, 2003; Shima et al., 1998; Winkel et al., 2008). Even though the concentration of these compounds in food products are generally below the taste threshold, they are still important as they can enhance other tastes, mask undesired taste, and provide background taste (Kaneko et al., 2011). Pyroglutamyl dipeptides can be produced from pyroglutamic acid and free amino acids by bacterial enzymes found in *L.*

helveticus, L.delbrueckii subsp. *bulgaricus*, *L. delbrueckii* subsp.*lactis*, and *Streptococcus thermophilus* (Altamura et al., 1970; Mucchetti et al., 2002). Moreover, lactoyl amino acid was produced from lactic acid and free amino acid in the presence of live or lysed *L. rhamnosus* and *L. helveticus* (Sgarbi et al., 2013). Succinyl amino acids, especially Suc-Arg and Suc-Glu from soy sauce, have umami taste, which may result from the arginine catabolic pathway (Frerot & Chen, 2013). Meanwhile, succinyl transferase from *Aspergillus oryzae* may also contribute to the generation of succinyl amino acids (Frerot & Chen, 2013). However, the study of amino acid derivatives in food is limited and more research is needed to understand the metabolic pathway of amino acid derivatives (Sgarbi et al., 2013). In bread, amino acid derivatives have never been reported.

Also, it is already known that some lactobacilli can produce glutathione reductase, which can convert oxidized glutathione (GSSG) in cereal protein to reduced glutathione (GSH). Glutathione imparts kokumi sensation and also affects the gluten network; it may therefore contribute to bread texture and taste (Jaensch et al., 2007; Ueda et al., 1997). By comparison of the isogenic mutant (Δgsh) and wild type strain, the impact of GSH on the quality of bread can be determined.

The effects of kokumi peptides or glutamate are determined by salt level. For example, at lower salt concentrations (1%), the additional kokumi peptides or glutamate not increase the salty taste of bread. However, with 2% salt there was a significant difference in salty taste if bread had different concentrations of kokumi dipeptides or glutamate. It is already known that there is synergistic interaction between MSG and NaCl. Also, it is well established that kokumi peptide can enhance salty taste. However, it is unclear how NaCl affects the perception of umami and kokumi taste. This knowledge could help to

understand the interaction between taste compounds and to find the optimal ingredient ratio. Therefore, further investigation is warranted on the taste-taste interaction.

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Appendix A

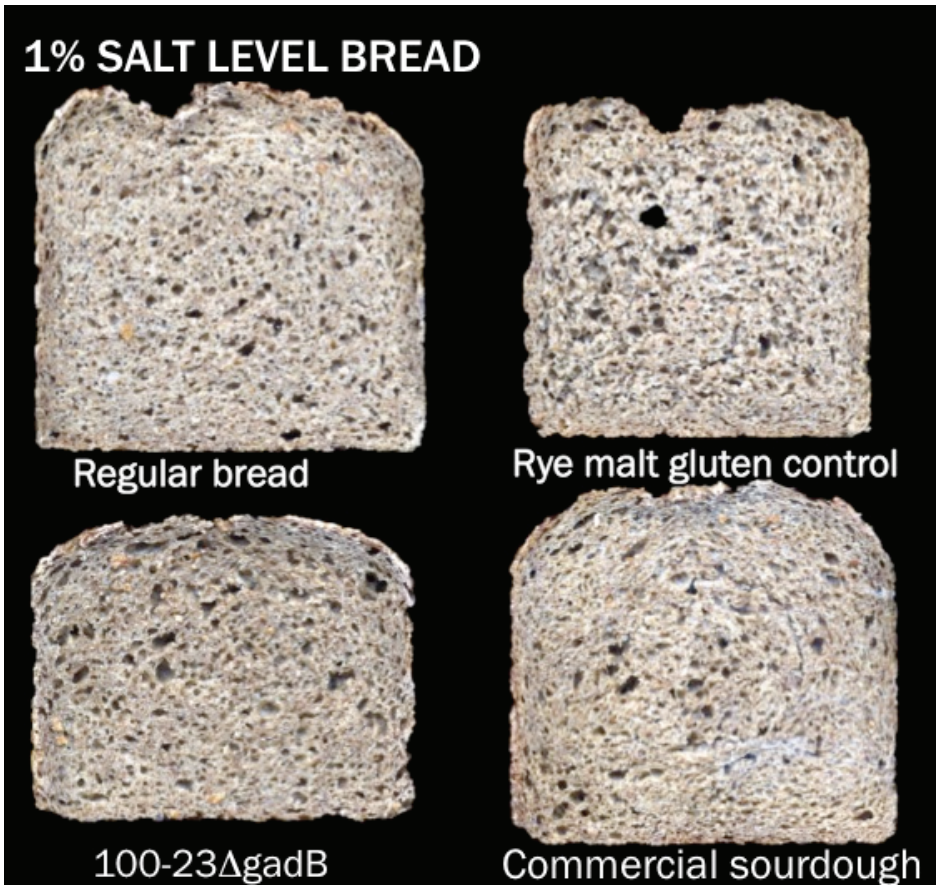

Figure A-1 The online supplementary material. Zhao et al.

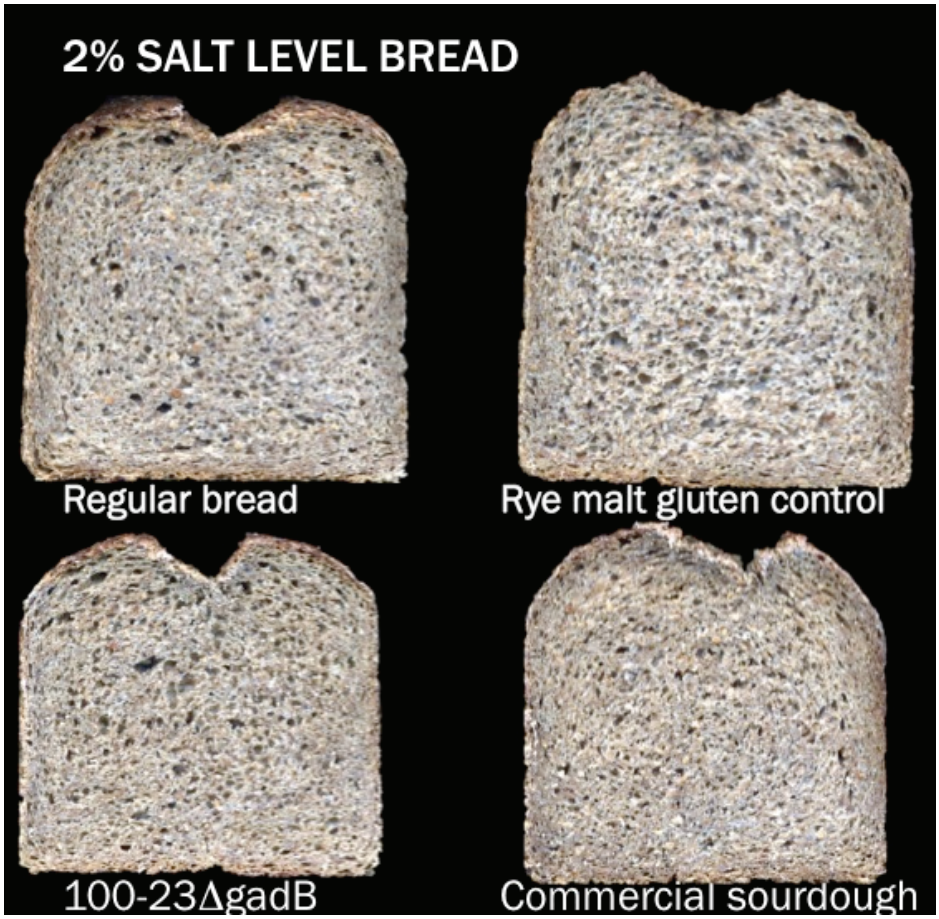

Figure A-2 The online supplementary material. Zhao et al.

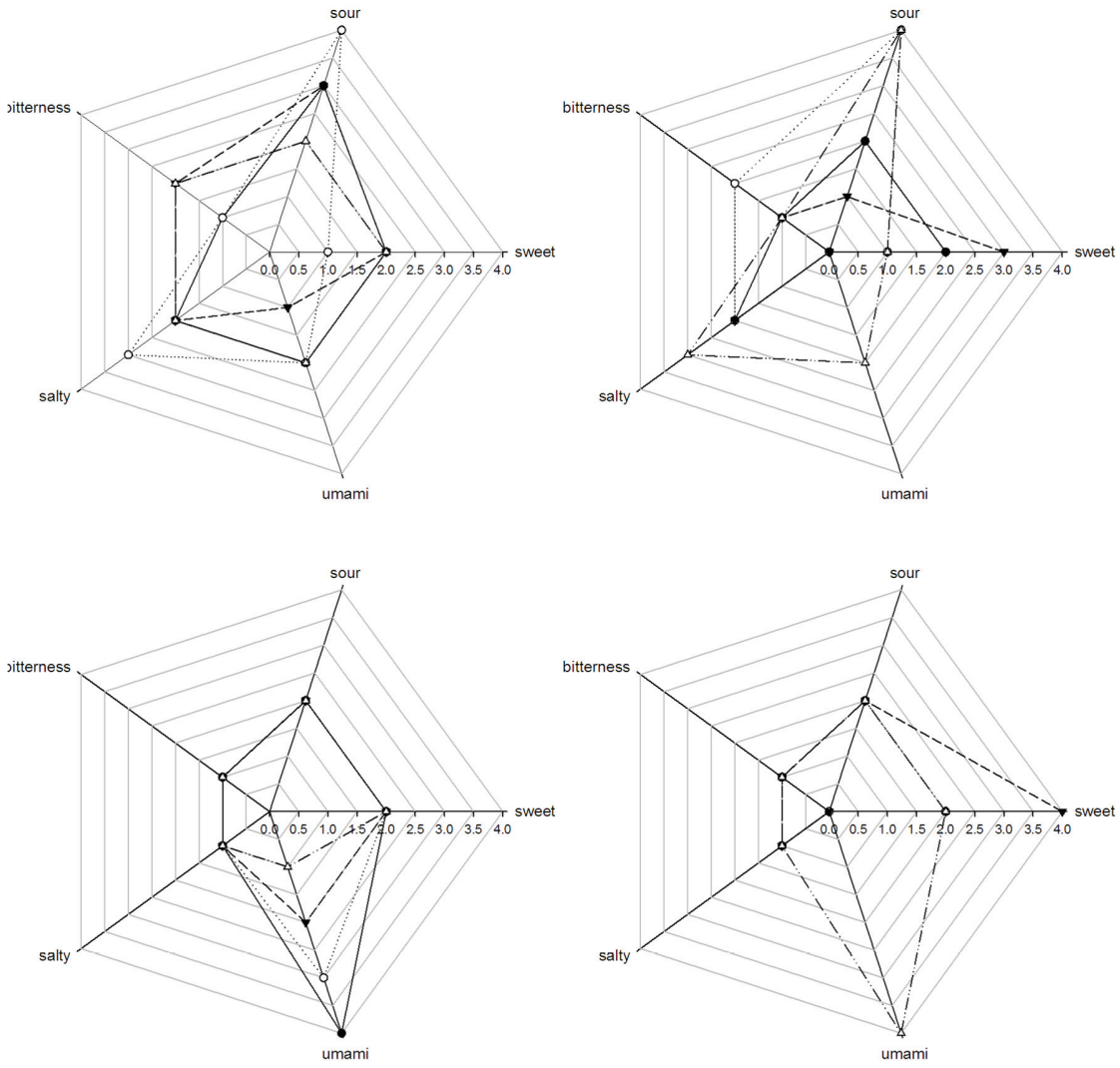

Figure A-3 The online supplementary material. Zhao et al.

Appendix B

Table B-1 pH and cell count of the sourdough fermented by different strains from 0 h to 96 h.

	TMW 1.106	**LTH5448**	**100-23**	**100-23 DgadB**	**Chemically acidified**
			pH		
0 H	6.05±0.02	6.095±0.02	6.095±0.06	6.10±0.02	3.38±0.03
24 H	3.57±0.02	3.55±0.01	3.6±0.00	360±0.01	3.65±0.02
48 H	3.46±0.12	3.61±0.02	3.65±0.01	3.66±0.01	3.99±0.02
72 H	3.71±0.02	3.72±0.00	3.735±0.01	3.71±0.00	3.77±0.01
96 H	3.81±0.03	3.83±0.03	3.79±0.01	3.80±0.02	3.97±0
			cell count		
0 H	1.00E+07	1.00E+07	1.00E+07	1.00E+07	1.00E+03
24 H	1.80E+09	1.90E+09	1.75E+09	8.90E+08	1.00E+03
48 H	5.95E+08	5.20E+08	2.05E+08	3.95E+08	7.50E+04
72 H	2.20E+09	1.45E+09	1.50E+09	6.40E+08	7.50E+04
96 H	6.40E+08	7.33E+08	6.10E+08	4.45E+07	1.00E+04

Table B-2 Concentration of γ-glutamyl dipeptides (μmol/kg) in sourdough fermented with different ingredients.

	Glu-Glu	Glu-Ile	Glu-Leu	Glu-Met	Glu-Phe	Glu-Val
			L.reuteri 100-23 OH			
Rye malt+ Fungal protease+MTG	6.72±2.08[d]	0.91±0.91[c]	3.78±3.78	1.78±0.11[ef]	NA	NA
Rye + Fungal protease+MTG	9.33±0.10[c]	NA	1.61±1.61	1.68±0.01[f]	NA	0.72±0.10
			L.reuteri 100-23 48 H			
Rye malt+ Fungal protease+MTG	8.04±0.13[d]	3.08±1.99[b]	4.17±0.61	3.15±0.58[c]	0.07±0.01	0.98±0.50
Rye + Fungal protease+MTG	9.68±0.31[c]	6.78±0.63[a]	3.04±0.65	2.51±0.14[de]	NA	4.87±3.89
			L.reuteri 100-23 96 H			
Rye malt+ Fungal protease+MTG	8.02±0.99[d]	0.64±0.64[c]	4.52±0.15	2.77±0.24[d]	0.07±0.01	1.78±0.22
Rye + Fungal protease+MTG	9.68±0.88[c]	1.93±0.44[bc]	3.60±0.51	2.37±0.04[de]	NA	3.95±4.30
			L.reuteri 100-23 ΔgadB OH			
Rye malt+ Fungal protease+MTG	5.99±2.14[d]	0.97±0.97[c]	3.90±3.90	1.69±0.01[f]	NA	0.76±1.07
Rye + Fungal protease+MTG	7.65±0.27[d]	NA	1.58±1.58	1.68±0.01[f]	NA	0.42±0.60
			L.reuteri 100-23 ΔgadB 48 H			
Rye malt+ Fungal protease+MTG	8.95±0.11[c]	4.97±1.64[a]	5.89±1.56	3.20±0.03[c]	0.08±0.01	0.51±0.07
Rye + Fungal protease+MTG	8.55±0.15[d]	5.97±0.17[a]	3.50±0.28	2.42±0.22[de]	NA	1.45±0.28
			L.reuteri 100-23 ΔgadB 96 H			
Rye malt+ Fungal protease+MTG	13.17±4.95[b]	0.01±0.01[c]	3.33±1.58	2.56±0.32[d]	NA	NA
Rye + Fungal protease+MTG	13.17±4.95[a]	1.00±0.45[c]	1.29±0.40	2.45±0.12[de]	NA	0.89±1.26

[a]The different letter in the same column means significant difference.

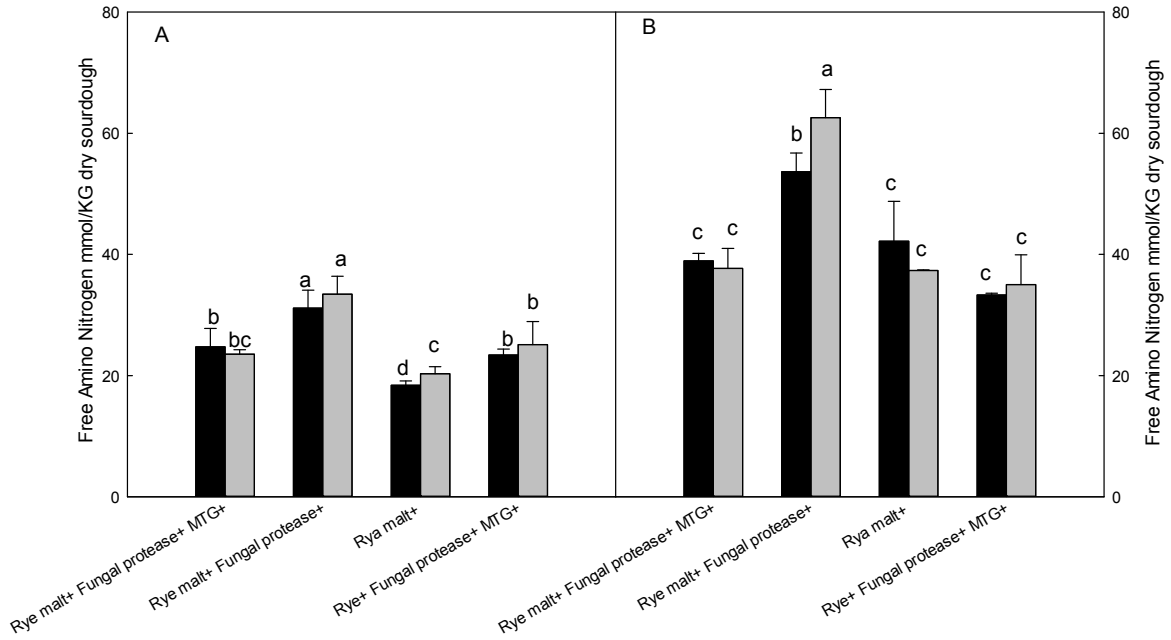

Figure B-1 Free amino nitrogen of different type of sourdough fermented by *L.reuteri* 100-23 (black bar) and *L.reuteri* 100-23 ΔgadB (grey bar)for 48 h(A) and 96 h(B) repectively. The different letter in the same figure means significant difference ($p<0.05$).