# Formation of taste-active metabolites in sourdough fermentation and the fermentation of plant-based cheese analogues

by

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# ABSTRACT

Fermentation is an oldest and effective method to generate taste-active compounds which contribute to food taste. The aim of this PhD dissertation was to investigate the synthetic mechanism of taste-active γ-glutamyl dipeptides during sourdough fermentation and the fermentation of plant-based cheese analogues, and their effect on taste of foods.

Kokumi γ-glutamyl dipeptides accumulate in sourdoughs. To understand the role of γ-glutamyl-cysteine ligases (Gcls) from lactobacilli in the formation of taste-active γ-glutamyl dipeptides in sourdough or sourdough bread, three Gcls from strains of *Lm. reuteri*, designated as Gcl1, Gcl2 and Gcl3, were characterized. In addition, sourdoughs were fermented with strains of *Lm. reuteri* or its Gcl-deficient mutant strains, subsequently used for the production of sourdough breads. The results showed that in enzymatic reaction with an individual amino acid, Gcl3 exhibits the broadest substrate specificity among three Gcls and all three Gcls were inactive to Val, Asp and His. All three Gcls of *Lm. reuteri* with a mixture of amino acids were most active to Cys among 12 amino acids. Gcl1 exhibited a stronger activity with hydrophobic amino acids Ile, Leu, and Phe when compared to Gcl2 and Gcl3. In sourdough fermentation, the accumulation of kokumi γ-glutamyl dipeptides was attributed to the combined activity of cereal enzymes, Gcls of lactobacilli and other microbial enzymes in lactobacilli. Although several γ-glutamyl dipeptides were synthesized during sourdough fermentation, baking processing strongly influenced the concentrations of γ-glutamyl dipeptides in bread. The effect of γ-glutamyl dipeptides in taste of bread remain to be investigated further.

Consumer demand for plant-based cheese analogues is increasing but challenges remain to improve the flavor and quality. The study described in thesis investigated microbiological and physicochemical impacts of seed germination and fermentation with *Bacillus* spp. on proteolysis

in plant-based cheese analogues and the contribution of adjunct cultures on the accumulation of kokumi γ-glutamyl dipeptides in the fermentation of plant cheese analogues. Bacilli but not seed germination enhanced proteolysis of plant-based cheese analogues and release of glutamate during ripening. *Lp. plantarum* and *Lc. lactis* served as starter cultures for plant milk acidification; *Lv. hammesii*, *Ff. milii*, or *Lt. buchneri* was assessed as an adjunct culture for the ripening of plant cheese analogues. During ripening of plant-based cheese analogues, *Lc. lactis* and *Lp. plantarum* were inactivated. Cell counts of *Lv. hammesii* remained stable over 45 d of ripening while *Ff. milii* and *Lt. buchneri* grew slowly. The combined activity of seed germination, *Bacillus* spp., and adjunct culture lactobacilli contributed to the synthesis of kokumi γ-glutamyl dipeptides in plant-based cheese analogues.

Overall, this research revealed the synthetic mechanism of taste-active γ-glutamyl dipeptides in sourdough fermentation and the fermentation of plant cheese analogues, providing insights into the control of formation of taste-active compounds in food fermentations.

# PREFACE

This thesis is an original work by Jin Xie, which is written according to the guidelines provided by Faculty of Graduate Studies and Research of University of Alberta.

Chapter 2 has been published as Jin Xie and Michael Gänzle. (2023), "Microbiology of fermented soy foods in Asia: can we learn lessons for production of plant cheese analogues?", International Journal of Food Microbiology, 407: 110399. I was responsible for searching references, literature review and manuscript preparation. Dr. Michael Gänzle provided a lot of comments and contributed to concept formation and manuscript revision.

Chapter 3 has been published as Jin Xie and Michael Gänzle. (2021), "Characterization of γ-glutamyl cysteine ligases from *Limosilactobacillus reuteri* producing kokumi active γ-glutamyl dipeptides", Applied Microbiology and Biotechnology, 105: 5503–5515. I designed study, analyzed data, conducted the experiments and prepared the manuscript. Dr. Michael Gänzle contributed to experimental design and manuscript revision.

Chapter 4 is an experimental work prepared for submission as Jin Xie, Ziyi Zhao, and Michael Gänzle, "Contribution of γ-glutamyl-cysteine ligases from *Limosilactobacillus reuteri* to the formation of kokumi taste-active γ-glutamyl dipeptides in sourdough or sourdough bread", to Journal of Agricultural and Food Chemistry. I am responsible for conducting the experiments, analyzing data and writing the manuscript. Ziyi Zhao participated in the experiments on the bread sensory evaluation together with me. Dr. Michael Gänzle provided suggestions on experimental design and manuscript edits

Chapter 5 has been published as Jin Xie, Gloria Yap, David Simpson, Michael Gänzle. (2024), "The effect of seed germination and *Bacillus* spp. on the ripening of plant cheese analogues", Applied and Environmental Microbiology. I designed study, conducted experiments,

analysed data and wrote the manuscript. David Simpson set up nanopore technology in lab 2-50 and provided suggestions on data analysis of sequencing. Gloria Yap performed experiments on the effect of *Bacillus amyloliqueficiens* on the ripening of plant cheese analogues togerther with me. Dr. Michael Gänzle contributed to the hypothesis development, experimental design and manuscript revision.

Chapter 6 is an experimental work prepared for submission as Jin Xie and Michael Gänzle, "Selection of cultures for the ripening of plant cheese analogues" toFood Microbiology. I designed study, performed experiments and wrote the manuscript. Dr. Michael Gänzle contributed to experimental design and the editing of manuscript.

# DEDICATION

*I would like to dedicate this PhD thesis to my beloved parents, Mr. Shaohong Xie and Mrs. Weixing Li, and my brother, Tao Xie. Thanks for your endless love, support and help in my life.*

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# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

LAB: Lactic acid bacteria

Gcl: γ-Glutamyl cysteine ligase

GshAB: Bifunctional γ-glutamyl-cysteine ligase/glutathione synthetase

mMRS: Modified deMan-Rogosa-Sharpe

LB: Luria-bertani

VRBG: Violet red bile glucose

Log: Logarithmic

CFU: Colony forming units

OD: Optical density

IPTG: Isopropyl-β-D-thiogalactopyranoside

NDA: 2,3-Naphthalenedicarboxaldehyde

L-BSO: L-Buthionine-sulfoximine

SSA: 5-sulfosalicylic acid

GSH: Glutathione

EDTA: Ethylenediaminetetraacetic acid

ATP: Adenosine triphosphate

MSG: Monosodium glutamate

DNS: 3, 5-Dinitrosalicylic acid

DTT: Dithiothreitol

IAM: Iodoacetamide

HPLC: High-performance liquid chromatography

MS: Mass spectrometry

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

MRM: Multiple reaction monitoring

HPAEC-PAD: High-performance anion-exchange chromatography with pulsed amperometric detection

ANOVA: Analysis of variance

BLAST: Basic local alignment search tool

NCBI: National Center for Biotechnology Information

RT-qPCR: Real-time quantitative polymerase chain reaction

rpm: Revolutions per minute

# Chapter 1. General Introduction and Thesis Objectives

## 1.1 Introduction

Lactic acid bacteria (LAB) dominate the microbiota of a majority of food fermentations but also cause food spoilage (Gänzle, 2015). LAB are defined as a heterogeneous group of gram-positive, cocci-shaped or rod-shaped, catalase-negative, non-spore-forming, fermentative, and facultative anaerobic bacteria that produce lactic acid as a main end-product of carbohydrate metabolism (Holzapfel and Wood, 2014). Phylogenetically, LAB belong to the phylum Firmicutes, class Bacilli, order *Lactobacillales* (Holzapfel and Wood, 2014). On the basis of fermentation characteristics, the order *Lactobacillales* can be grouped into homofermentative and heterofermentative organisms. The order of *Lactobacillales* comprises five families: *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Streptococcaceae*, and *Lactobacillaceae* (Holzapfel and Wood, 2014; J. Zheng et al., 2020). Food fermenting lactic acid bacteria mainly include *Lactobacillaceae* and two genera each in the *Enterococcaceae* and *Streptococcaceae*. *Tetragenococcus halophilus* in the genus *Tetragenococcus* (Udomsil et al., 2011) and *Enterococcus faecalis* and *Enterococcus faecium* in the genus *Enterococcus* (Jeong et al., 2015) serve as food fermenting organisms. Of the genus *Streptococcus*, only *Streptococcus thermophilus* is safe for the use of starter culture in dairy production (yogurt and cheese), other *Streptococcus* species include pathogenic organisms. *Lactococcus lactis* and *Lc. cremoris* are the only lactococci used in food fermentations, these organisms also produce nisin (Holzapfel and Wood, 2014). The family *Lactobacillaceae* constitute non-pathogenic organisms and many have a safe tradition of use in food fermentations including dairy production, meat fermentations, sourdough, vegetable fermentations (sauerkraut, kimchi), wine (malolactic fermentation), beer (wort fermentation, sour beers) (Gänzle, 2015). Food fermenting *Lactobacillaceae* include species from 20 genera

including *Lactobacillus*, *Pediococcus*, *Weissella*, *Oenococcus*, *Leuconostoc*, and the 15 new genera *Companilactobacillus*, *Schleiferilactobacillus*, *Lacticaseibacillus*, *Latilactobacillus*, *Liquorilactobacillus*, *Ligilactobacillus*, *Lactiplantibacillus*, *Furfurilactobacillus*, *Paucilactobacillus*, *Limosilactobacillus*, *Fructilactobacillus*, *Acetilactobacillus*, *Levilactobacillus*, *Secundilactobacillus* and *Lentilactobacillus* (Qiao et al., 2022; J. Zheng et al., 2020). In food fermentations, the metabolism and/or conversion of carbohydrates, peptides, amino acids, phenolic compounds and lipids by LAB determines food quality including sensory properties, shelf life and safety of food products (Gänzle, 2015; Gänzle et al., 2023b).

Taste, an important determinant of food quality, comprises six basic tastes: sweet, sour, bitter, salty, umami (Nelson et al., 2001) and oleogustus (Gaillard and Kinnamon, 2019), which are sensed by oral taste receptors. In addition to taste-active compounds, other taste-modulating compounds impact oro-gustatory taste perception (Deepankumar et al., 2019). The taste sensation described as "mouthfulness", "thickness", and "continuity" is designated as kokumi taste and is mediated by kokumi active compounds (Nishimura et al., 2019). γ-Glutamyl peptides with an N-terminal γ-L-glutamyl residue, when present in micromolar concentrations, exhibit kokumi activity by modulating the activity of Calcium-sensing receptors (CaSRs) in taste cells. CaSR is involved in the signal transduction from taste receptors to the brain (Ohsu et al., 2010). In addition, γ-glutamyl peptide (γ-Glu-Val) shows the *in vivo* anti-inflammatory activity; this activity is also mediated by interaction with CaSRs (H. Zhang et al., 2015; Zhang et al., 2016). γ-Glutamyl peptides occur in plants such as garlic, onions, wheat and edible beans (Ueda et al., 1990; Sarwin et al., 1992; Ueda et al., 1994; Dunkel et al., 2007) and yeast extract (Liu et al., 2015). These peptides were also identified in fermented foods including soy sauce, ripened cheese and sourdough bread (Kuroda et al., 2013; Toelstede et al., 2009; Zhao and Gänzle, 2016). In

sourdough fermentation or cheese ripening, the concentrations of kokumi active γ-glutamyl peptides can be increased by enzymatic activity of raw materials and microbial activity (Hillmann et al., 2016; Toelstede and Hofmann, 2009; Yan et al., 2018).

Microbial enzymes involved in the synthesis of γ-glutamyl peptides include γ-glutamyl transferase/transpeptidase (GGT, EC 2.3.2.2, Suzuki et al. 2007), glutaminase (EC 3.5.1.2, Nandakumar et al. 2003) and γ-glutamyl-cysteine ligase (EC 6.3.2.2, Kino et al., 2007). γ-Glu-Cys ligases catalyze the first and rate-limiting step in glutathione (GSH) biosynthesis and synthesize γ-Glu-Cys in an ATP-dependent reaction by ligating the γ-carboxyl group of glutamate to cysteine (Kelly et al., 2002). Gcls occur in many prokaryotes and in virtually all eukaryotes. Structurally, Gcls from bacteria, non-plant eukaryotes (mammals and yeast) and plants function as monomeric enzymes, heterodimeric enzymes with intramolecular disulfide bonds, and homodimeric enzymes with two intermolecular disulfide bonds (Galant et al., 2011), respectively. Gcl domains of bifunctional γ-glutamyl-cysteine ligase/glutathione synthetase (GshAB) from *Escherichia coli*, *Streptococcus agalactiae* and *Clostridium acetobutylicum* have been characterized *in vitro* and these synthesize γ-glutamyl peptides (Kelly et al., 2002; Kino et al., 2007). Heterofermentative lactobacilli frequently harbor *gcl* genes; genomes of strains in the genera *Levilactobacillus*, *Limosilactobacillus* and *Lentilactobacillus* frequently encode for several *gcl* genes (Yan et al., 2018). However, Gcls from lactobacilli have not been characterized yet and their characteristics, in particular their activity for the synthesis of γ-glutamyl peptides, remain unknown.

Sourdough, a cereal dough, has been used for bread production more than 14,000 years (Arranz-Otaegui et al., 2018). The family *Lactobacillaceae* dominate the microbiota of sourdough fermentation. In sourdoughs that are used as a sole leavening agent, heterofermentative *Fructilactobacillus sanfranciscensis* is one of dominant fermentation microbes and is often

associated with *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Leuconostoc* spp. or *Weissella* spp.(Gänzle and Zheng, 2019). Sourdoughs that are fermented to achieve the acidification include *Lactobacillus* and *Limosilactobacillus* species as dominant microbes (Gänzle and Zheng, 2019). In spontaneous sourdoughs, *Lp. plantarum* and *Lm. fermentum* are most frequently identified (Gänzle and Zheng, 2019). Metabolic activities of sourdough microbes contribute to bread quality. The formation of lactate and acetic acid by carbohydrate metabolism of LAB improves the flavor of bread, modulates the activity of the cereal enzymes, and extends mold-free shelf life (Gänzle, 2014; Quattrini et al., 2019). The exopolysaccharides synthesized by glucansucrases of *Lm. reuteri*, *Leuconostoc* spp. and *Weissella* spp. improve bread volume and texture (Galle et al., 2010). Hydration of unsaturated fatty acids to hydroxyl-fatty acids by *Lv. hammesii* and *Lp. plantarum* enhances the mold-free shelf life (Black et al., 2013). The accumulation of γ-glutamyl peptides and the release of amino acids such as glutamate in sourdoughs fermented with *Lm. reuteri* strains may contribute to the taste of bread; the accumulation γ-glutamyl peptides was strain-specific (Yan et al., 2018; Zhao et al., 2015; Zhao and Gänzle, 2016) (Figure 1.1). However, the synthetic mechanism of γ-glutamyl peptides in sourdough fermentation with lactobacilli remain unclear. In particular, the role of Gcls from lactobacilli in biosynthesis of γ-glutamyl peptides in sourdough fermentation remains to be investigated.

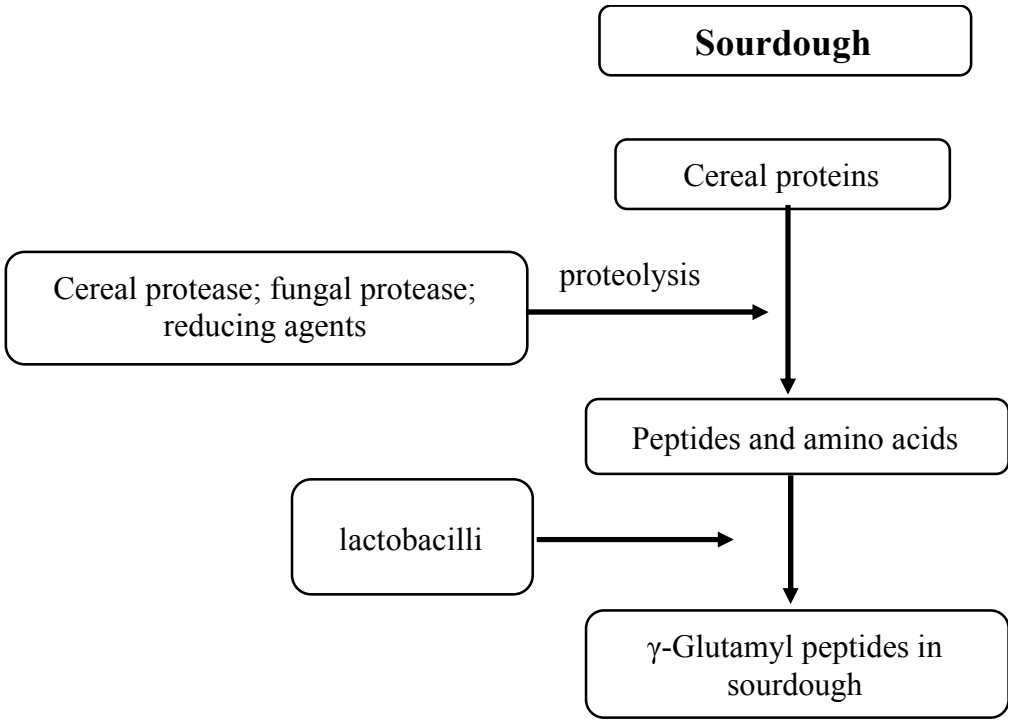



**Figure 1.1.** Overview on the formation of γ-glutamyl peptides in sourdough fermentation.

LAB are also dominant microbes in cheesemaking and contribute to the flavor of cheese (Parente et al., 2017). Two stages of fermentation including milk acidification and cheese ripening are involved in the production of ripened cheese. Starter cultures for acidification predominantly include *St. thermophilus* with *Lactobacillus delbrueckii* or *Lactobacillus helveticus*, or *Lc. lactis* and *Lc. cremoris* associated with *Leuconostoc* spp.(Parente et al., 2017). The metabolism of citrate by *Leuconostoc* spp. or specific strains of *Lc. lactis* contributes to the formation of cheese eye and the accumulation of diacetyl and acetate. The release of intracellular peptidases by autolysis of lactic starters accumulates free amino acids which are flavor compounds or precursors of flavor compounds. The adjunct cultures occurring in cheese ripening include yeasts (*Geotrichum candidum* and *Debaryomyces hansenii*), molds (*Penicillium camemberti* and *Penicillium roqueforti*), and bacteria (*Brevibacterium*, *Corynebacterium*, *Staphylococcus*, *Micrococcus*, *Propionibacterium* spp., and nonstarter lactic acid bacteria (NSLAB)) (Irlinger et al., 2017).

NSLAB encountered in cheese ripening are derived from raw milk or natural whey culture and environment, including *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*, *Lentilactobacillus buchneri*, *Latilactobacillus curvatus*, *Lp. plantarum*, *Lv. brevis*, *Pediococcus* spp., *Leuconostoc* spp., and *Enterococcus* spp. (Irlinger et al., 2017). In ripening, yeasts, molds, and aerobic bacteria mainly grow on cheese surface to develop the appearance and flavor of cheese. NSLAB growing in the cheese matrix contribute to the texture and flavor of cheese by producing EPS, aldehydes, ketones, and alcohols (Irlinger et al., 2017). Kokumi γ-glutamyl peptide accumulate during cheese ripening. Their accumulation is attributed to γ-glutamyl transpeptidase activity from raw milk or from *P. roqueforti* rather than lactobacilli strains lacking genes encoding for γ-glutamyl transpeptidase or Gcls (Hillmann et al., 2016; Toelstede and Hofmann, 2009).

Plant-based cheese analogues from plant materials are considered as healthy and sustainable foods, incentivising researchers to develop novel plant cheese products. Fermentation as an oldest but safe and effective tool has been used to improve sensory properties of plant-based cheese analogues (Tangyu et al., 2019). As a novel plant food, the microbiology of fermented plant-based cheese analogues is not well studied. The development of plant-based cheese analgues was informed by the traditional use of cultures in meat and cheese fermentations and the selection of starter cultures often defaults to *Lp. plantarum* and *La. casei* (Mefleh et al., 2021). LAB associated with plants or occurring in spontaneous plant fermentation may function as starter cultures for the fermentation of plant-based cheese analogues, but their selection and potential use in plant-based cheese analogues remain to be investigated. Traditional fermentation of plant protein foods, in particular fermented soy foods in Asia, may provide scientific guidance for design of fermentation processes and for selection of starter cultures.

In summary, although microbiology of fermented plant-based cheese analogues are understudied, microbiology in sourdough and cheese fermentation are well-known. The selection of functional starter culture for the production of plant-based cheese analogues may be guided by traditional plant food fermentation. LAB as dominant microbes contribute the flavor of fermented plant foods such as sourdough bread, but their contributions on the formation of taste-active γ-glutamyl peptides remain unclear. Therefore, this thesis generally aimed to well understand the synthetic mechanism of kokumi γ-glutamyl peptides in sourdoughs and fermented plant-based cheese analogues.

## 1.2 Hypothesis

Lactobacilli, particularly Gcls from lactobacilli, contribute to the accumulation of kokumi γ-glutamyl dipeptides in sourdough fermentation and the fermentation of plant-based cheese analogues.

Kokumi γ-glutamyl dipeptides synthesized in fermentation contribute to the taste of bread and plant cheese analogues.

## 1.3 Objectives

1) To describe the production process and microbiology of fermented soy foods in Asia to provide knowledges for the production of fermented plant-based cheese analogues (Chapter 2).

2) To characterize Gcls from *Lm. reuteri* producing kokumi γ-glutamyl dipeptides (Chapter 3).

3) To evaluate the contribution of Gcls from *Lm. reuteri* to the formation of kokumi γ-glutamyl dipeptides in sourdough or sourdough bread (Chapter 4).

4) To develop a novel methodology to enhance the proteolysis that generates substrates for the biosynthesis of kokumi γ-glutamyl dipeptides in the fermentation of plant-based cheese analogues (Chapter 5).

5) To select starter cultures and evaluate the contribution of Gcls from lactobacilli on the accumulation of kokumi γ-glutamyl dipeptides during ripening of plant-based cheese analogues (Chapter 6).

# Chapter 2. Microbiology of fermented soy foods in Asia: can we learn lessons for production of plant cheese analogues?

## 2.1 Introduction

Fermentation is a traditional, low cost and effective unit operation in food production. It transforms agricultural raw materials into functional and flavored foods through desirable microbial growth and enzymatic activity (Marco et al., 2021). Fermentation is also considered as an effective tool to improve food safety through the reduction of anti-nutritive or even toxic compounds, and through fermentative production of antimicrobial products including organic acids, alcohols, and bacteriocins by that control spoilage organisms and pathogens (Gänzle, 2020; Ross et al., 2002). The characteristics and quality of final fermented products depends on the dominant microorganisms or core microbiota. Microorganisms commonly used for food fermentation include lactic acid bacteria, acetic acid bacteria, yeasts and filamentous fungi (Marco et al., 2021). Globally, fermented protein foods are prepared from soy or legumes, or from animal protein. In the European tradition of food fermentations, fermented protein foods are exclusively produced with animal proteins, i.e. meat, milk or fish (Gänzle, 2022). The current trend towards replacing animal proteins with plant proteins to increase the sustainability of food production, and to meet the demand of vegan or vegetarian consumers also necessitates to develop plant-based analogues of fermented animal protein foods (Grossmann and McClements, 2021). To date, the development of these fermented plant-protein foods was informed by the traditional use of cultures in meat and cheese fermentations, or the selection of starter cultures defaults to *Lactiplantibacillus plantarum* and *Lacticaseibacillus casei* (Mefleh et al., 2021). However, traditional fermentation of plant protein foods, particularly fermented foods prepared from soy beans, may provide an

additional knowledge base for design of fermentation processes, and for selection of starter cultures.

Legumes are among of the most important agricultural crops. Multiple traditional fermented foods employ legumes as main substrate and, among legumes, soy beans are most commonly used as substrate for traditional fermented protein foods (Gänzle, 2022). Soy beans were domesticated several thousand years ago in East Asia and has been introduced to other parts of the world less than 500 years ago. It has a high protein content, about ~ 40% and a well-balanced amino acid composition (Etten et al., 1959; Medic et al., 2014). In addition, soy proteins exhibit good functional properties, including gelling formation, emulsification, water-holding, and foaming (Wolf, 1970). Thus, soy beans are an important source of plant proteins. In addition to the high content of proteins, soy beans also contain other nutrients including diverse carbohydrates and lipids as well as isoflavones (Medic et al., 2014). In East Asian countries, a variety of fermented soy foods has been traditionally prepared and consumed as staple foods or as condiments; examples include *douchi*, *natto*, *tempeh*, and *sufu*. Of these, *douchi*, *natto*, and *tempeh* are whole soybean-fermented products while *sufu*, a soft creamy "cheese-like" product, is fermented soybean curd (Lite, 2005).

Fermented soy foods are produced with diverse fermentation microbes. *Natto* is fermented with *Bacillus subtilis* biovar. Natto (Hosoi and Kiuchi, 2008), but *Rhizopus* spp. is a common starter culture for tempeh-making (Hartanti et al., 2015). Traditional fermented soy foods such as *douchi*, *sufu*, and *tempeh* are made using spontaneous fermentation, or with pure starter cultures to initiate the fermentation, followed by the maturation (Han et al., 2001b; Lite, 2005; Nout and Rombouts, 1990). Their microbial communities significantly differ depending on the processing method even though they are the same type of fermented soy products, subsequently influencing

the flavor and quality of final products. There are two *douchi* products, *Aspergillus*-type Liuyang *douchi* and *Mucor*-type Yongchuan *douchi*, that exhibit remarkable differences in the microbiota composition and flavor (H. Yang et al., 2019; Zhang et al., 2021). Although the fermentation process, microbial compositions and their function on the flavor formation have been described, there are few studies on summarizing their microbial diversity and microbial contributions to the flavor and quality of final fermented soy products.

The review summarizes the production process and microbiology of five fermented soy foods in Asia, including *douchi*, *natto*, *tempeh*, and *sufu* as well as stinky tofu, evaluates the potential contribution of microbes to the flavor and quality of final products, and discusses the question whether these traditional fermented products provide guidance for the development of plant-based analogues of plant-based cheeses and fermented meats.

## 2.2 Fermentation of soy beans

### 2.2.1 *Douchi*

*Douchi*, a traditional fermented soybean product has been consumed in China as a protein source or seasoning, and has been used in Chinese traditional medicine (Chen et al., 2007; Lite, 2005; Lite et al., 2002). An overview on the production of *Mucor*-type and *Aspergillus*-type *Douchi* is shown in Figure 2.1.

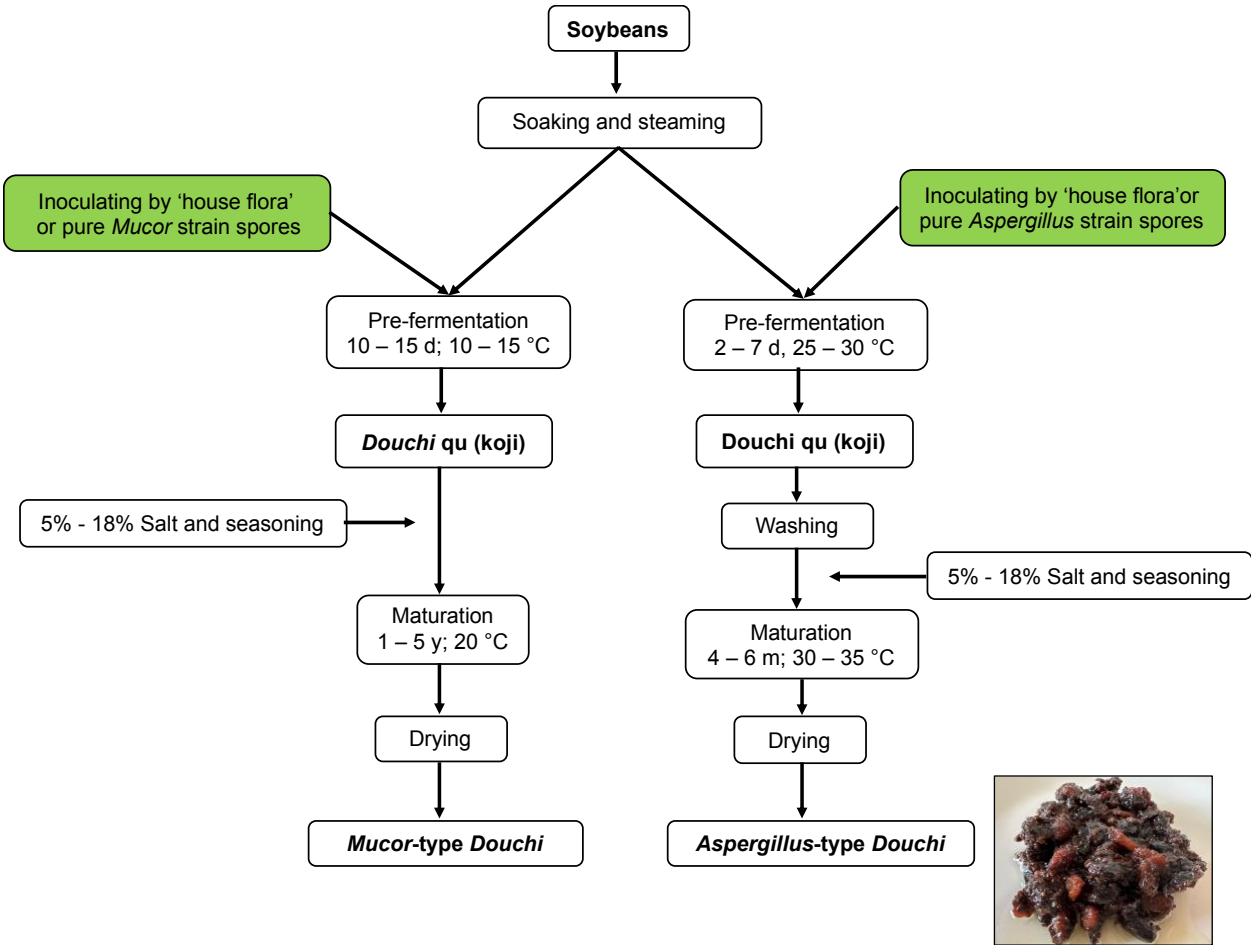



**Figure 2.1.** Flowchart for the fermentation of *Mucor*-type and *Aspergillus*-type *Douchi*. Red coloring indicates a heating step that inactivates vegetative microorganisms; green coloring indicates the inoculation with starter cultures; blue coloring indicates addition of ingredients that alter intrinsic factors which influence microbial growth. The seasoning or dressing mixture contains 5 – 18% salt (Endo et al., 2014; He et al., 2016; Lite, 2005; Zhang et al., 2007) and additionally includes mixture of dry ground ginger, shallots or garlic (Zhang et al., 2007), a small amount of sugar, and selected spices such as capsicum paste (Lite, 2005), 5% fermented rice and 5% liquor (He et al., 2016), Chinese prickly ash, fresh hot pepper paste, and dry hot pepper powder (Liu et al., 2012). The moisture content of the final product ranges from 35 to 50% (Lite, 2005); the final pH ranges from pH 6.5 to 6.9 (Wang et al., 2010a). The photo of douchi at the lower left represents a product purchased at a local supermarket in Edmonton, Alberta, Canada.

The production of *douchi* involves a two-stage fermentation process: koji-making or pre-fermentation and maturation or post-fermentation (Figure 2.1). According to the microbes in the koji-making phase, *douchi* can be grouped into three types: *Aspergillus*-type, i.e. Baoxiangyuan

and Liuyang *douchi*, *Mucor*-type, i.e. Yongchuan *douchi*, and *Bacillus*-type, i.e. Qingyang *douchi and* Longnan *douchi* (Chen et al., 2007; Lite, 2005; Zhang et al., 2018). *Mucor douchi* is fermented at a lower temperature but for a longer fermentation time when compared to *Aspergillus* type *douchi* (Figure 2.1) (Lite, 2005). The salt content of *Aspergillus*-type Baoxiangyuan and Liuyang *douchi* ranges from 4 to7% (w/w) (T. Chen et al., 2011b; Yang et al., 2016; Zhang et al., 2022), which are low-salt *douchi* products, but other *Aspergillus*-type *douchi* is produced with up to 16% (w/w) NaCl (Lite, 2005; Zhang et al., 2007). *Mucor*-type *douchi* is commonly produced as high-salt *douchi* with a salt content of more than 12% (He et al., 2016; Hu et al., 2012).

*Aspergillus*-type *douchi* is most widely produced. *Aspergillus*-type *douchi* is made in Southern China, e.g. Hunan, Jiangxi, Sichuan, and Guangdong provinces (Q.-C. Chen et al., 2011; Chen et al., 2012; Fan et al., 2009). *Aspergillus* spp. and *Bacillus* spp. were identified as dominant microorganisms during pre-fermentation; *Aspergillus* strains originate from inoculation with starter cultures while *Bacillus* spp. originate from the 'house flora' or the raw material (T. Chen et al., 2011a, 2011b; Zhang et al., 2007). In the pre-fermentation step, *Aspergillus* and *Bacillus* spp. produce hydrolytic enzymes including proteases, cellulases, amylases, and lipases and thus generate substrates for other microorganisms in the subsequent *douchi* maturation (Hu et al., 2022; Pel et al., 2007; Zhang et al., 2007). To prevent the formation of bitterness and astringency of the final products, the extend of hydrolysis is controlled by washing and addition of brine (Lite, 2005). *Douchi* is subsequently maturated in a brine mixture of salts and seasonings without addition of starter cultures; the microbial communities of *douchi* and the flavor of the end products varies depending on the change of environmental conditions. The bacterial and fungal communities listed in Table 2.1 were identified as the dominant microbiota during maturation of *Aspergillus*-type

*douchi* or in mature Baoxiangyuan and Liuyang *douchi*, which contain 4 – 7% NaCl (Chen et al., 2012, 2014, 2022; T. Chen et al., 2011b; Li et al., 2018; Yang et al., 2019, 2016).

**Table 2.1.** Fermentation microorganisms identified in *Aspergillus*-type- and in *Mucor*-type *douchi*.

| ***Douchi*** | | **Fermentation Microorganism** |
|---|---|---|
| *Aspergillus*-type *douchi* | Pre- | *Aspergillus oryzae, Bacillus subtilis, Aspergillus niger, Aspergillus egypticus* |
| | Post- | *Anaerosalibacter, Pseudomonas, Bacillus, Staphylococcus, Enterococcus, Lactococcus, Weissella, Pediococcus, Pichia, Aspergillus, Lichtheimia, Petromyces, Rhizopus, Penicillium, Candida, Saccharomyces,* and *Debaryomyces* |
| *Mucor*-type *douchi* | Pre- | *Mucor* spp.*, Actinomucor elegans* |
| | Post- | *Tetragenococcus halophilus, Zygosaccharomyces rouxii, Candida versatilis, Staphylococcus, Bacillus, Aspergillus,* and *Penicillium* |

With information from: (Chen et al., 2007, 2012, 2014; T. Chen et al., 2011a, 2011b; He et al., 2016; Li et al., 2018; Lite, 2005; Yang et al., 2019, 2016; Zhang et al., 2007b, 2021).

*Mucor*-type *douchi* is mainly produced in South-Western China (Wang et al., 2021; Yu et al., 2022; Zhang et al., 2021) In pre-fermentation, *Mucor* species are inoculated as starter cultures (Lite, 2005) and also secrete hydrolytic enzymes. *Mucor qu* is prepared at lower temperature with slower fermentation compared to *Aspergillus qu* (Figure 2.1). For *Mucor*-type *douchi*, *Mucor qu* is mixed directly with salts and seasonings without washing, followed by the secondary fermentation. During post-fermentation phase of Yongchuan *douchi*, the bacterial genera *Bacillus*, *Enterobacter*, and *Pseudomonas*, and the fungal genera *Penicillium* and *Aspergillus* were identified as dominant members of fermentation microbiota (Zhang et al., 2021).

Bacteria-type *douchi* is commonly produced in North China including Beijing and the Shandong and Gansu provinces. It is usually generated through the spontaneous fermentation with "house flora". Thus, the specificities of traditional bacteria-type *douchi* production vary depending on the environmental conditions. Zhang et al. (Zhang et al., 2018) characterized the microbial

diversity of two bacteria-type *douchi* products and found that the microbial communities differed. The bacterial genera *Bacillus* and *Ignatzschineria* and the fungal genera *Pichia* and *Candida* were present in both *douchi* products.

The accumulation of tastants and volatile odorants imparts fermented *douchi* with a unique odor and taste. The content of free amino acids increased gradually during fermentation (Zhang et al., 2007) and most of the volatiles are generated in the post-fermentation stage (H. Yang et al., 2019). Because the optimum water activity for the Maillard reaction, 0.5 - 0.7 (Pereyra Gonzales et al., 2010), matches the water activity of *douchi* during drying, Maillard products that are formed during drying may additionally contribute to the flavor formation of *douchi* (L. Liu et al., 2022). Different types of *douchi* exhibit a different composition of volatiles due to the distinct composition of fermentation microbiota during post-fermentation. In *Aspergillus*-type Liuyang *douchi*, the volatiles phenylethyl alcohol, phenethyl acetate, isoamyl acetate, 2-methyl-butanal, ethyl 2-methylbutyrate, 2,6-dimethylpyrazine, 1-octen-3-ol, 2-pentyl furan, benzeneacetaldehyde and phenethyl butyrate were identified (Q.-C. Chen et al., 2011; Chen et al., 2021). Naturally maturated *Mucor*-type *douchi* contained a higher diversity of volatile compounds including esters, phenols, and pyrazines compared to *Mucor*-type *douchi* inoculated by defined starters (He et al., 2016), likely reflecting that more diverse fermentation microbiota generally result in a more diverse and intense flavor profile. The use of co-cultures which consist of the salt tolerant *Tetragenococcus halophilus, Zygosaccharomyces rouxii* and *Candida versatilis* accelerated the formation of volatiles (He et al., 2016). Wang et al. (Wang et al., 2021) identified 10 compounds present with high odor activity values in *Mucor*-type Yongchuan *douchi*, including diacetyl, dimethyl trisulfide, acetic acid, acetylpyrazine, 3-methylvaleric acid, 4-methylvaleric acid, 2-methoxyphenol, maltol, γ-nonanolactone, eugenol and phenylacetic acid. *Aspergillus*-type *douchi*

had more phenols and pyrazines while *Mucor*-type *douchi* contained more esters and acids (Wang et al., 2010b). *Mucor*-type Yongchuan *douchi* matured for 10 month contained a higher content of the umami-tasting glutamic acid and aspartic acid and a lower content of bitter-tasting amino acids such as isoleucine, leucine and arginine when compared to *Aspergillus*-type Yongchuan *douchi* that was matured for one month (Zhang et al., 2021). Esters that impart floral and fruity odors, including ethyl isovalerate, isoamyl acetate, and ethyl phenylacetate, were also present in higher quantities in the *Mucor*-type Yongchuan *douchi* when compared to *Aspergillus*-type Yongchuan *douchi* (Zhang et al., 2021).

### 2.2.2 *Natto*

Natto, a Japanese fermented soybean food, is categorized into three types based on the production methods and microorganisms used, including *yukiwari-natto*, *hama-natto*, and *itohiki-natto*. *Itohiki-natto* (hereafter shortened to "natto") is fermented with *Bacillus subtilis* biovar. Natto is the most widely consumed product in Japan (Hosoi and Kiuchi, 2008; Reddy et al., 1983; Ruiz Sella et al., 2021). The procedure for *natto*-making is shown in Figure 2.2.

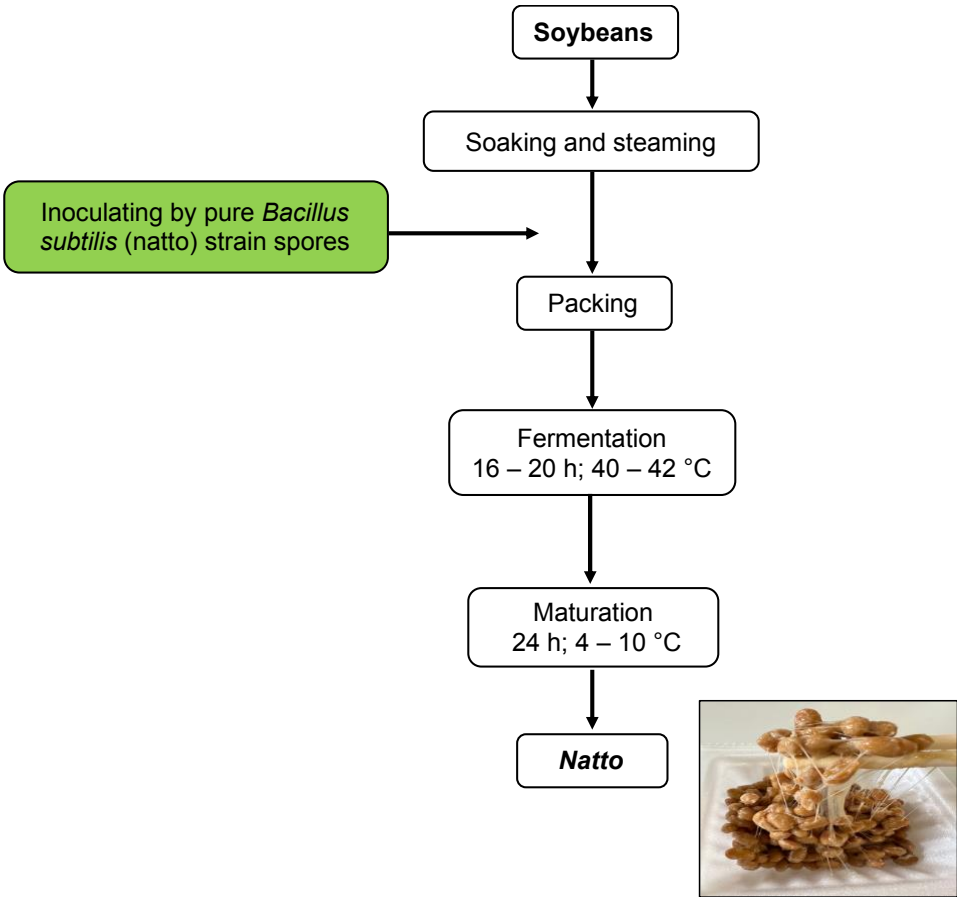



**Figure 2.2.** Flowchart for fermentation of *natto*. Red coloring indicates a heating step that inactivates vegetative microorganisms; green coloring indicates the inoculation with starter cultures. The moisture content of the product is 59.5% (aw 0.93 to 0.97) (Kim et al., 2012), the pH ranges from 4.9 to 7.5 (Kim et al., 2012; Tsai et al., 2007). The surface of *natto* is covered with slime or mucus that consists of 58 % of γ-polyglutamic acid and of 40% of polysaccharides (Saito et al., 1974). The photo of *natto* at the lower left represents a product purchased at a local supermarket in Edmonton, Alberta, Canada.

Natto strains are able to impart *natto* with the characteristic flavor and the sticky texture after fermentation. They have a very similar genome structure to other *B. subtilis* strains that are not suitable for *natto*-making but additionally require biotin for growth (Kubo et al., 2011). The sticky material on the *natto* surface consists mainly of poly-γ-glutamate (γ-PGA) and polysaccharides (Saito et al., 1974). The texture of *natto* is dependent on γ-PGA; the amount of γ-PGA produced, in turn, relates to the available carbon sources and the concentration of L-glutamate

(Shih and Van, 2001). More γ-PGA was produced by *Bacillus subtilis* biovar. Natto using a medium with citric acid, ammonium sulfate and a lower concentration of L-glutamate (Kunioka, 1995). The genetic determinants of *Bacillus* species that relate to their use in food fermentations including *natto* fermentation were recently reviewed (Li et al., 2023b).

The flavor characteristics of *natto* depends on the fermentation conditions, the raw material, and *natto* strains that are used (Wei et al., 2001). A storage temperature of more than 15 °C promoted the production of ammonia and branched-fatty acids during maturation, leading to an undesirable ammonia smell (Kanno and Takamatsu, 1987). Thus, storage temperatures of less than 10 °C are used to obtain good quality *natto* (Kada et al., 2008). Soy beans with a higher sugar content but a lower protein and oil content generated *natto* products with superior sensory properties (Yoshikawa et al., 2014). Natto strains secret protease, especially the serine protease nattokinase, to hydrolyse proteins into peptides and amino acids (Ichishima et al., 1986; Ju et al., 2019; Lan et al., 2020). Natto strains also express levansucrase to synthesize the polysaccharide levan from sucrose or raffinose (Bersaneti et al., 2018; Shih et al., 2010, 2005). Pyrazines produced by *B. subtilis* biovar. Natto strains are key contributor to the characteristic odour of natto (Kłosowski et al., 2021; Kosuge et al., 1971; Liu et al., 2018a; Sugawara et al., 1985). Key aroma compounds in natto include 2,3-butanedione, 5-methyl-2-hexanone, 3-hydroxy-2-butanone, 2-nonanone, furaldehyde, acetic acid, 2-ethyl butyric acid, ethyl acetate, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, 3,5-dimethyl-2-ethylpyrazine, 2,3,5,6-tetramethylpyrazine, 2,3,5-methyl-6-ethylpyrazine, and benzaldehyde; of these, the pyrazines exhibited the highest odor activity values (Liu et al., 2018b; Tanaka et al., 1998).

### 2.2.3 *Tempeh*

*Tempeh* (or *tempe*) is a mold-fermented soybean food which is commonly consumed in Indonesia as snack or as staple food (Nout and Rombouts, 1990). Other legumes, cereal grains and processing by-products have been also used for the tempeh production. Flowchart on the production of tempeh is shown in Figure 2.3. Tempeh-making involves a two-stage fermentation: the natural fermentation by lactic acid bacteria during soybean soaking and fungal fermentation by *Rhizopus* spp. starter cultures (Figure 2.3).

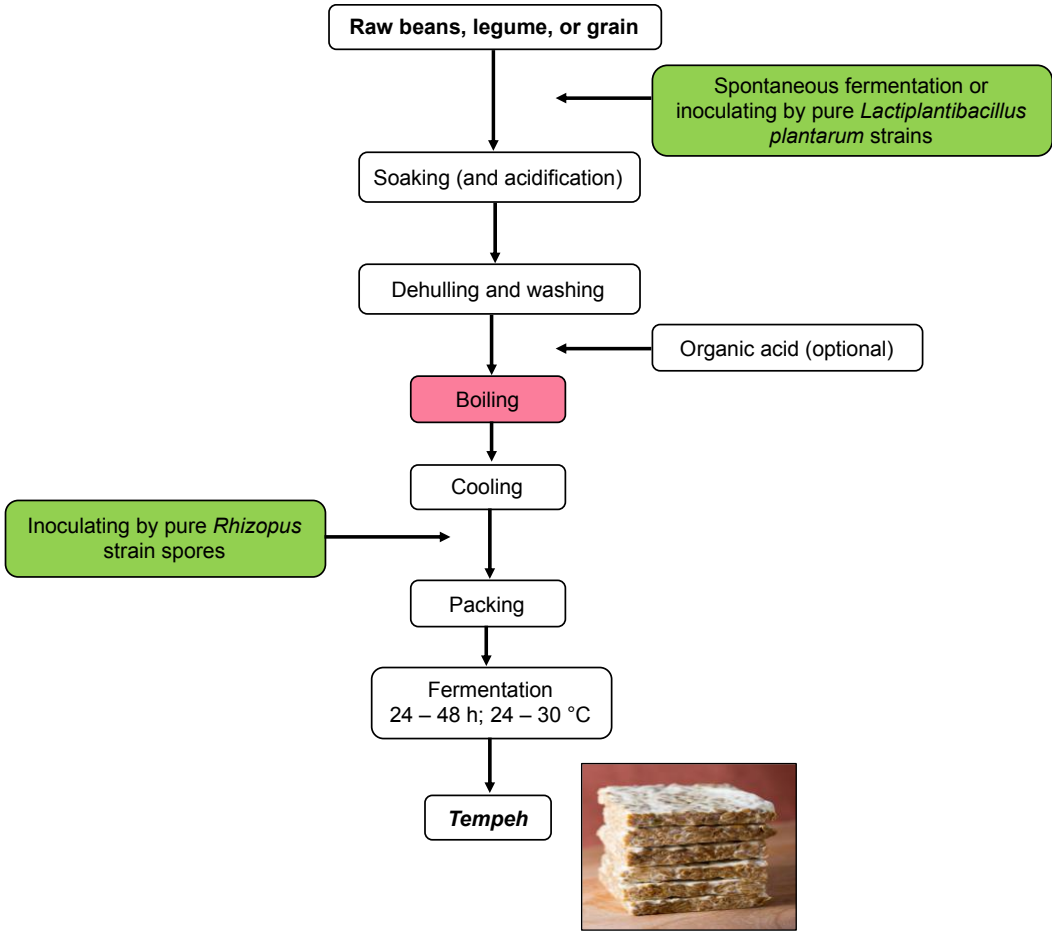



**Figure 2.3.** Flowchart for the production of *tempeh*. Red coloring indicates a heating step that inactivates vegetative microorganisms; green coloring indicates the inoculation with starter cultures; blue coloring indicates addition of ingredients that alter intrinsic factors which influence microbial growth. The moisture content of the final product is about 58%, the pH ranges from 6.6 to 7.1 (Sparringa and Owens, 1999). The photo of *tempeh* at the lower left was available under a Creative Commons License on Aug 12, 2023 on https://en.wikipedia.org/wiki/Tempeh.

In the first-stage, spontaneous fermentation, microorganisms shown in Table 2.2 were identified, in which *Lacticaseibacillus casei*, *Enterococcus faecium* and *Staphylococcus epidermidis* are dominant microbiota responsible for the acidification during the soaking of soy beans, where the pH value drops from 6.5 to 4.5 (Mulyowidarso et al., 1989). To increase the reliability of the acidification by lactic acid bacteria during soaking, the addition of lactic or acetic acids to the soaking water (Nout et al., 1985), back-slopping of the soaking water, or use of *Lactiplantibacillus plantarum* as starter culture was used (Nout et al., 1987b). *Leuconostoc fallax*, *Pediococcus pentosaceus*, and *Weissella cibaria* were identified as dominant members of bacterial communities after 10 back-slopping cycles at 30 °C (Yan et al., 2013). Acidification to pH values ranging from pH 3.5–5.0 (Yoneya, 2004) inhibits the growth of potential pathogenic and spoilage microorganisms including *Staphylococcus aureus* (Nout et al., 1988) and *Bacillus cereus* (Nout et al., 1987a) but does not impact growth of *Rhizopus* if the pH remains above 3.5.

**Table 2.2.** Fermentation microorganisms identified in *tempe* or *tempeh.*

| Tempeh | | Fermentation Microorganism |
|---|---|---|
| *Tempeh* | Pre- | *Klebsiella pneumoniae, Klebsiella ozaenae, Enterobacter cloacae, Enterobacter agglomerans, Citrobacter diversus, Bacillus brevis, Pichia burtonii, Candida diddensiae, Rhodotorula rubra, Lacticaseibacillus casei, Enterococcus faecium, Staphylococcus epidermidis,* and *Streptococcus dysgalactiae,* and co-inoculant *Lactiplantibacillus plantarum* |
| | Post- | *Rhizopus* spp. (*R. arrhizus, R. delemar, R. microsporus* and *R. stolonifer*), *Bacillus pumilus, Bacillus brevis, Streptococcus faecium, Lacticaseibacillus casei, KIebsiella pneumoniae Enterobacter cloacae, Micrococcus varians, Trichosporon beigelii, Clavispora (Candida) lusitaniae, Candida maltose, Candida intermedia,Yarrowia lipolytica, Mucor indicus and Mucor circinelloides ,* and/or co-inoculants: lactobacilli*, Citrobacter freundii, Klebsiella pneumoniae,* and *Propionibacterium freudenreichii* |

With information from (Mogessie Ashenafi and Busse, 1991; Feng et al., 2005; Hartanti et al., 2015; Keuth et al., 1994; Mulyowidarso et al., 1990, 1989; Nout et al., 1987a; Samson et al., 1987; Signorini et al., 2018; Sjamsuridzal et al., 2021; Wiesel et al., 1997)

After soaking, the hulls of soy beans are removed, followed by a cooking process that softens the matrix and eliminates most undesirable microorganisms. In the second-stage of fermentation, *Rhizopus* spp. such as *R. arrhizus*, *R. delemar*, *R. microsporus* and *R. stolonifer* are dominant (Dwidjoseputro and Wolf, 1970; Hartanti et al., 2015; Sjamsuridzal et al., 2021). In addition to *Rhizopus* spp, bacteria and yeasts have frequently been isolated from tempeh (Table 2.2) (Ashenafi and Busse, 1991b; Mulyowidarso et al., 1990; Samson et al., 1987). The use of co-inoculant lactobacilli in tempeh fermentation prevents the growth of undesirable microorganisms and generate a high quality product (Nout et al., 1987a). *Citrobacter freundii*, *K. pneumoniae* or *Propionibacterium freudenreichii* have also been investigated as a co-inoculants to increase the yield of vitamin B12 in tempeh fermentation (Keuth and Bisping, 1994; Signorini et al., 2018; Wiesel et al., 1997).

Fresh tempeh appears as a firm cake covered with white mycelium and a meaty, mushroom-like and nutty flavor (Hachmeister and Fung, 1993). *Rhizopus* spp. strains as dominant fermenters produce a variety of glycosyl hyrolases, proteases and lipases to break down polysaccharides, proteins and lipids (Nout and Rombouts, 1990). Amino acids and peptides impart umami taste (Amin et al., 2020). 2-Methylpropanal, 1-octene-3-ol and 3-(methylthio)propanal were determined as the major aroma compounds in tempeh fermented by *R. oligosporus* NRRL 2710 (Jeleń et al., 2013). The use of the co-inoculant *Saccharomyces cerevisiae* additionally contributed to the formation of alcohol, ester, styrene, phenol, and maltol compared to the regular fermented tempeh (Kustyawati et al., 2017).

## 2.3 Fermentation of precipitated soy proteins

### 2.3.1 *Sufu (furu)*

*Sufu* (*Furu* or *Dou-furu*), a fermented soybean curd product is a soft, flavoured and creamy cheese-like product that is consumed as staple food in China. The production of *sufu* involves two-step fermentation processes: pre-fermentation or *pehtze*-making and post-fermentation or *sufu* ripening (Figure 2.4). According to the microbes that are present in *pehtze*-making, *sufu* can be grouped into mold-fermented and bacteria-fermented sufu (Han et al., 2001b).

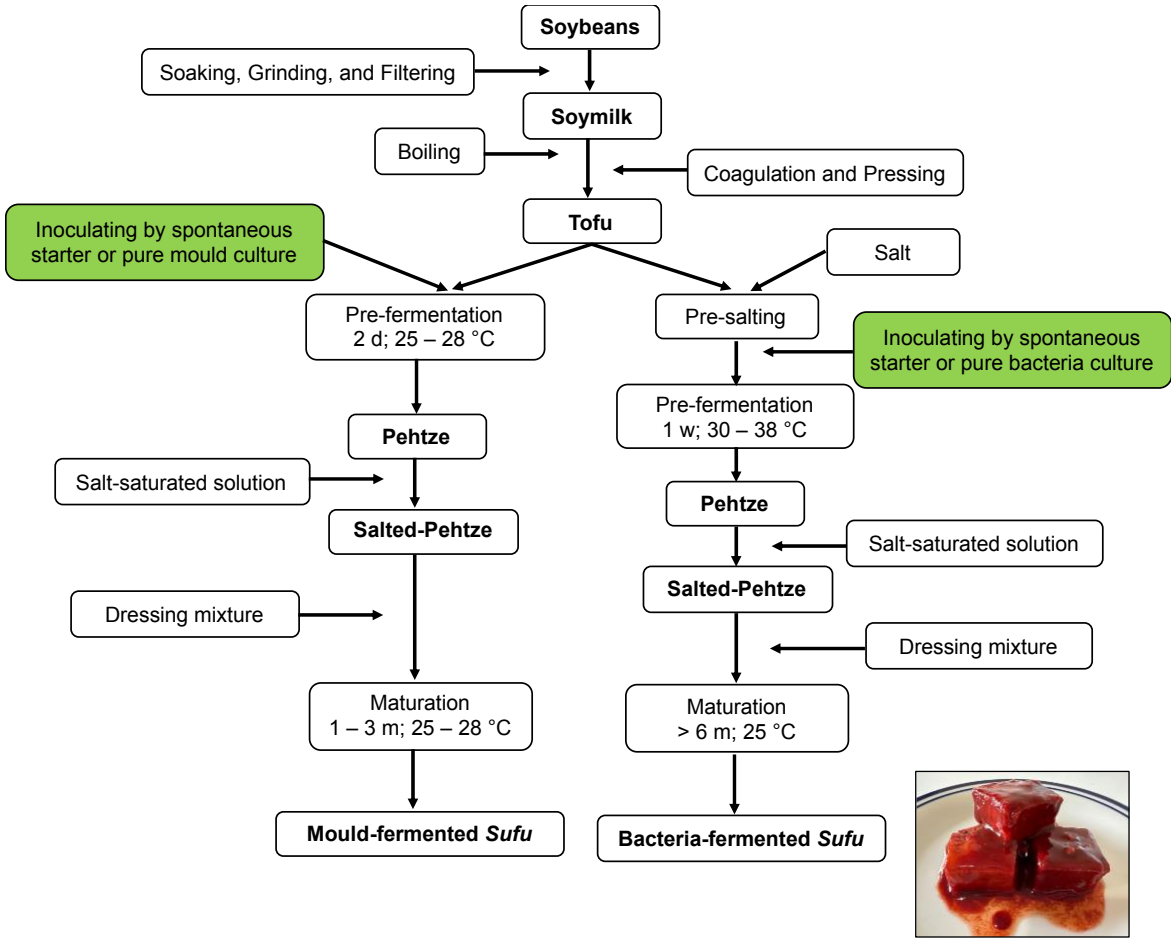



**Figure 2.4.** Flowchart for the production of mould-fermented and bacteria *sufu.* Red coloring indicates a heating step that inactivates vegetative microorganisms; green coloring indicates the inoculation with starter cultures; blue coloring indicates addition of ingredients that alter intrinsic factors which influence microbial growth. The final products have a moisture content of 58-73% (Han et al., 2001b, 2001a), a salt content of 6-15% (w/w) (Han et al., 2001a, 2004a), an ethanol content of 0-7% (v/v) (Han et al., 2004a) and a pH of 5.25-7.45 (Han et al., 2001a). The dressing mixture contain one or more of the following: Red sufu, salt (10-14%), angkak (2%), alcoholic beverage (final alcohol content, 5-12% v/v), sugar (5-10%), soybean paste (3-5%), Chiang (wheat-based miso, 3%); the dressing mixture for white sufu is similar but contains no angkak (red rice fermented with *Monascus purpureus*); Grey sufu, soy whey, salt (14%). The photo of *sufu* at the lower left represents a product purchased at a local supermarket in Edmonton, Alberta, Canada.

Dominant microorganisms during *sufu* ripening are shown in Table 2.3. The molds *Actinomucor* spp., *Mucor* spp., and *Rhizopus* spp. are commonly used as pure starter cultures for the *pehtze*-making of mold-fermented sufu (Cheng et al., 2011; Han et al., 2004a, 2001b; Huang

et al., 2018; X. Li et al., 2021), which produce proteases (Chou and Hwan, 1994; Han et al., 1988). Strains of *Micrococcus* spp. and/or *Bacillus* spp are also used as starter cultures for the production of bacteria-fermented sufu (Bao et al., 2020; Han et al., 2004a), of these two, *Bacillus* spp. produce extracellular proteases (Li et al., 2023b).

**Table 2.3.** Fermentation microorganisms identified in *sufu*.

| Type of product | | Fermentation Microorganism |
|---|---|---|
| Mould-fermented sufu | Pre- | *Actinomucor* spp.*, Mucor spp.* and *Rhizopus spp. (Actinomucor elegans, Actinomucor taiwanensis, Actinomucor repens, Mucor racemosus, Mucor Wutongqiao, Mucor circinelloides, Mocur hiemalis, Mucor flavus, Rhizopus microsporus* var. *microspores,* and *Rhizopus oryzae)* |
| | Post- | **Pure culture fermentation:** *Enterobacter, Acinetobacter, Lactococcus, Tetragonococcus, Bacillus, Enterococcus, Streptococcus, Macrococcus, Monascus and Aspergillus;* **Spontaneous fermentation:** *Bacillus, Enterococcus, Lactococcus, Leuconostoc, Lactobacilli, Weissella, Enterobacter, Tetragenococcus, Trabulsiella, Sphingobacterium, and Stenotrophomonas, Sterigmatomyces, Debaryomyces, Tausonia, Pichia, Candida, Geotrichum, Fusarium, and Actinomucor* |
| Bacteria-fermented sufu | Pre- | *Bacillus* spp. or *Micrococcus spp.* |
| | Post- | **Spontaneous fermentation:** *Enterococcus avium, Enterococcus faecalis, Staphylococcus carnosus, Leuconostoc mesenteroides, Staphylococcus saprophyticus, Streptococcus lutetiensis, Kocuria rosea, Kocuria kristinae, Bacillus pumilus, Bacillus cereus group*[*]*,* and *Bacillus subtilis* |

[*]The organism was identified by sequencing of 16 rRNA genes, which does not differentiate between *B. cereus*, *B. thuringiensis*, *B. anthracis* and closely related species. With information from (Feng et al., 2013; Han et al., 2001b; He and Chung, 2020; Huang et al., 2018; K. Li et al., 2022; X. Li et al., 2021; Liang et al., 2019; Tan et al., 2020; Wan et al., 2020; Xu et al., 2020).

Flavor formation occurs predominantly in the ripening stage of *sufu* fermentation and is highly dependent on the dressing mixtures (Figure 2.4) and the activity of microorganisms (He et

al., 2022; He and Chung, 2020; Huang et al., 2018; Song et al., 2021; Yao et al., 2021). The final salt content of *sufu* ranges from 10 to 14%, imparting a salty taste to *sufu* (Han et al., 2001b). In ripened *sufu*, free amino acids accumulate and umami-tasting glutamic acid was the most abundant (Han et al., 2004b; Kim et al., 2011; Li et al., 2010; Xie et al., 2018; Yao et al., 2021). Ethyl 2-methylpropanoate, diacetyl, ethyl butanoate, ethyl 2-methylbutanoate, 3-(methylthio)propanal, ethyl 3-phenylpropionate, 2-heptone, ethyl pentanoate, ethyl hexanoate, ethyl heptanoate, ethyl octanoate, benzaldehyde, and phenylethanol contribute to the aroma of red sufu (Chung, 2000; Chung et al., 2005; Wang et al., 2019). (E,E)-2,4-Decadienal, ethyl hexanoate, eugenol, methional, ethyl 2-methylbutyrate, (E,E)-2,4-nonadienal, and 1-octen-3-ol were identified as aroma compounds with high odour activity value in white *sufu* (He et al., 2020). Production of volatile compounds including hexadecenoic acid ethyl ester, methoxy acetic acid pentyl ester, benzene propanoic acid ethyl ester, ethyl 9-hexadecenoate, ethyl oleate, ethanol, 3-methyl-1-butanol, 5-methoxy-1-pentanol, and eugenol during ripening provided the typical flavor for bacteria-fermented Kedong *sufu* (Fan et al., 2019).

### 2.3.2 *Stinky tofu*

Stinky tofu (*choudoufu*), a popular Chinese snack, essentially consists of tofu seasoned with a fermented brine. The flowchart for making of stinky tofu is shown in Figure 2.5.

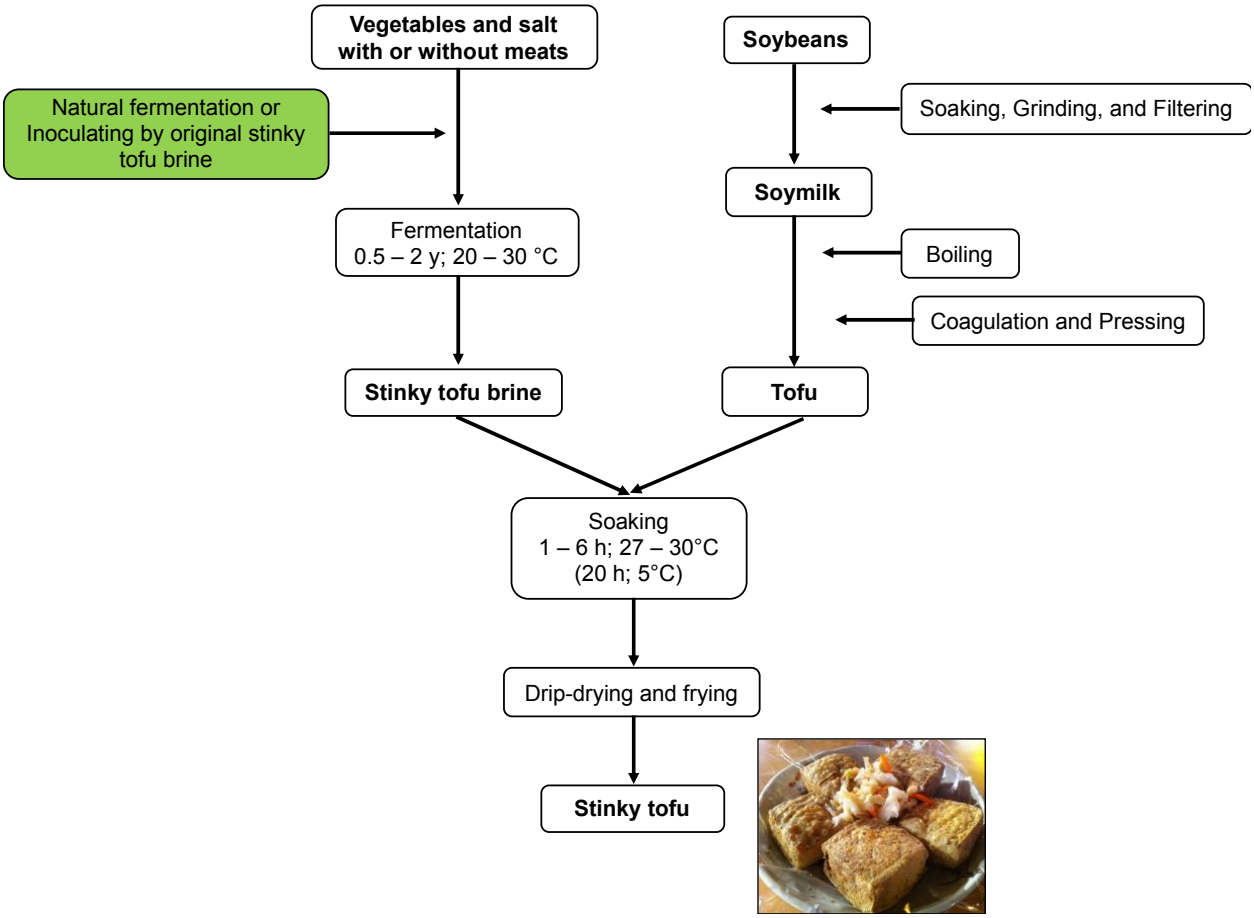



**Figure 2.5.** Flowchart for fermentation of stinky tofu (*choudoufu* or smelly tofu). Red coloring indicates a heating step that inactivates vegetative microorganisms; green coloring indicates the inoculation with starter cultures. pH of stinky tofu brines: 5.20~7.72 (Chao et al., 2008b), salt of stinky tofu brines: 1% (Chao et al., 2008b; Teng et al., 2004). The photo of stinky tofu at the lower left was available under a Creative Commons License on Aug 12, 2023 on https://en.wikipedia.org/wiki/Stinky_tofu.

The Mandarin word for stinky tofu (臭豆腐, *choudoufu*) is best translated as "smelly tofu" because the word "*chou"* has no negative connotation and is also used for strong but acceptable flavors. In contrast, the English word "stinky" is used to describe a negative perception of odor. The scientific literature, however, consistently refers to *choudoufu* as "stinky tofu" so this communication follows convention. The brine with the strong odour is produced by fermentation. Two formulas with different raw materials are commonly used, one consisting mainly of mixed

vegetables such as spiny amaranth, bamboo shoot and wax gourd with or without meat; the second additionally contains mashed tofu (Chao et al., 2008). Only few studies describe differences in the microbial communities in the two types of stinky brines. Also, microbes that are present during different fermentation phases are only partially investigated (Gu et al., 2018a). Lactic acid bacteria and the genus *Bacillus* are considered as dominant microbiota in the fermentation of stinky brines. Organisms of the genera *Enterococcus*, *Lactococcus*, *Streptococcus,* several genera of the *Lactobacillaceae* as well as *Bacillus* spp. such as *B. megaterium*, *B. polymyxa*, *B. pumilus*, *B. subtilis*, and *B. sphaericus* were identified in fermented stinky brines (Chao et al., 2008; 2010; Gu et al., 2018b; Lei et al., 2013; G. Liu et al., 2022). The lactic acid bacteria in stinky tofu brines overlap with core microorganisms in spontaneous vegetable fermentation (Ashaolu and Reale, 2020). The strains of the genus *Paraeggerthella* and *Eggerthella* involving the S-equol production were also isolated from brines of stinky tofu (Abiru et al., 2013).

Stinky tofu is categorized as alkaline fermented food where ammonia is produced from amino acids during fermentation. Ammonia formed during the fermentation of stinky brines results from the deamination of amino acids in neutral or alkaline conditions. Ammonia and $CO_2$ in the stinky brine permeates into the tofu during brining but evaporates during deep-frying or steaming process, imparting a fluffy and porous texture on the surface of stinky tofu (Teng et al., 2004). Volatile compounds imparting the characteristic flavor of stinky brines include camphene, caryophyllene, D-limonene, 3-(1,5-dimethyl-4 hexene)-6 methylene-cyclohexene, 5-(1,5-dimethyl-4-hexene)-2-methyl-1,3-cyclohexane), butanoic acid, pentanoic acid, heptanoic acid, octanoic acid, nonanoic acid, N-decanoic acid, dodecanoic acid, tridecanoic acid, hexadecenoic acid, and tetradecanoic acid, 2,6-dimethyl-2-octanol, butane, pentanoic acid-2,2,4-trimethyl-3-

carboxyisopropyl-isobutyl ester, 2-undecanone, dimethylamine, and sulfur hydrogen sulfide (Wang et al., 2020).

## 2.4 Similarities and difference of fermented soy products and cheeses

Focussing on the differences first, the substrate supply in soy or plant-based cheese analogues produced from soy or pulses along with other ingredients such as nuts or seeds and fats or oils differs substantially from the substrate supply in milk or cheeses. Milk contains lactose as sole source of fermentable carbohydrates and lactose metabolism is a major criterion for starter cultures (van de Guchte et al., 2006; Wels et al., 2019). Soybean and soybean curd contain more diverse carbohydrates, including fructose, glucose, sucrose, maltose, raffinose, stachyose and verbascose (Medic et al., 2014) and the ability of cultures to ferment raffinose-family oligosaccharides likely impacts acidification (Teixeira et al., 2012). In addition, plant-based cheese analogues contain starch and non-starch polysaccharides (Medic et al., 2014), which are completely absent in milk or cheese. Extracellular glycosyl hydrolases that degrade starch and non-starch polysaccharides thus impact the ripening. These enzymes are commonly produced by bacilli and mycelial fungi (Li et al., 2023b) but not by lactic acid bacteria or yeasts (Gänzle and Follador, 2012). Phytate hydrolysis as well as the degradation of other anti-nutritive components including raffinose-family oligosaccharides and lectins is relevant in fermentation of pulses and cereals but not in milk (Gänzle, 2020; Tsuji et al., 2015). Pulses, as any other plant material, contain phenolic compounds and the ability of lactic acid bacteria to hydrolyse glycosides of secondary plant metabolites or to convert phenolic acids to flavor volatiles or other bioactives will impact product quality (Gaur and Gänzle, 2023; C. Li et al., 2021). Last but not least, milk fat is composed of diverse fatty acids including C4 to C7 short chain fatty acids that are odor-active upon release from the triglycerides (McSweeney et al., 2020) but short chain fatty acids are

generally absent in plant oils. Conversely, unpasteurized plant substrates have lipoxygenase activity which oxidizes unsaturated fatty acids and generates the "beany" flavor of protein preparations from pulses. This flavor defect requires heterofermentative lactobacilli to reduce the odor-active aldehydes to alcohols with a much higher odor threshold (Sugahara et al., 2022).

The primary acidification cultures in cheese making and in fermentation of plant cheeses also differ. Cheese-making always involves primary acidification with a traditional back-slopped starter culture, or defined strain starter cultures derived from traditional cultures that contain *Streptococcus thermophilus* with *Lactobacillus delbrueckii* or *Lactobacillus helveticus*, or *Lactococcus lactis* and *Lactococcus cremoris* in association with *Leuconostoc* spp. (Parente et al., 2017). *L. delbrueckii* is highly specialized on lactose as substrate (van de Guchte et al., 2006) and unlikely to perform well in any plant fermentation. *Lc. lactis* and *Leuconostoc* spp. also occur in association with plants and in spontaneous plant fermentations and may be suitable for production of plant-based cheese analogues (Sooresh et al., 2023; Strafella et al., 2021). The traditional fermentation of soy (Tables 1 – 3) and other plant fermentations (Gänzle, 2022) relies on dozens of other species of lactic acid bacteria that may be more suitable for fermentation of plant-based cheese analogues than either lactococci with *Leuconostoc* spp. or the current default *Lp. plantarum* or *Lc. casei* (Mefleh et al., 2021). While the commercial availability of starter cultures dedicated to plant-based dairy and meat alternatives is increasing, these do not yet take advantage of the biodiversity of lactobacilli with potential use in plant-based cheese analogues. For example, the use of heterofermentative lactobacilli likely is a necessity to control the "beany" flavor caused by lipid oxidation products (Sugahara et al., 2022).

The succession of fermentation microbiota also differs in cheese ripening and traditional soy fermentations. Cheese making relies on acidification by lactic acid bacteria, followed by

ripening with diverse ripening cultures. Depending on the type of cheese, microorganisms during ripening include non-starter lactic acid bacteria, propionibacteria, *P. roqueforti* or the surface cultures *Penicillum camemberti* in association with yeasts including *Debaryomyces hansenii* and *Geotrichum candidum*. Bacterial red smear surface cultures include *Brevibacterium* spp. and *Corynebacterium* spp. in addition to a large diversity of other microbes (Fox et al., 2017; Irlinger et al., 2017; Wolfe et al., 2014). In traditional Asian pulse fermentations, this sequence is reversed; hydrolytic cultures including mycelial fungi and / or bacilli grow first, followed by growth of yeasts and lactic acid bacteria to develop the texture, taste and odor of the product (Figures 1 and 4). Because most lactobacilli do not express extracellular protease activities (Zheng et al., 2015a), a pre-fermentation with protease-producing microbes may be suitable for products that are composed of pasteurized ingredients.

The fermentation of cheeses and of traditional pulse fermentations is similar in one major aspect: Microbial metabolism converts sugars, proteins and lipids to taste-active compounds and odorants. The composition of ripening microbiota in cheeses and fermented soy food overlaps to some extend and includes e.g. *Lacticaseibacillus* spp. and *Lactiplantibacillus* spp.. Thus, the formation of tastants and odorants in cheeses and plant-based cheese analogues may involve comparable microbes, metabolic pathways, and flavor-active compounds.

Carbohydrate metabolism is an important way to produce flavor compounds. In cheese making, the flavor compounds such as acetate, diacetyl and acetoin are generated through the metabolism of lactose and citrate by the starter culture (Lo et al., 2018; McSweeney et al., 2017). The conversion of citrate to diacetyl has also been described for plant-based fermentations with addition of lemon or apple juice as a source of citrate (Comasio et al., 2021).

Proteolysis and catabolism of amino acids are the major process for the flavor formation of cheese (Yvon and Rijnen, 2001). The molds and some of lactic acid bacteria in cheese aging possess comprehensive proteolytic systems, which can accelerate cheese ripening and convert the peptides that are produced by protease and peptidase originating from milk, coagulant and starter cultures to small peptides and amino acids (Griffiths and Tellez, 2013; McSweeney, 2017). Some of peptides and amino acids directly contribute to the flavor of cheese; the bitter peptides produced in aging process provided ripened Cheddar cheese with a perception of bitterness (Karametsi et al., 2014), and glutamic acid generated in ripening phase imparts umami taste (Ganesan and Weimer, 2017; Toelstede and Hofmann, 2008). Proteolysis is also a critical source for the production of flavor compounds in fermented soy foods; glutamic acid was as the most abundant of free amino acids in ripened *sufu* and several hydrophobic amino acids such as Phe, Leu, and Val contributed to bitterness of *sufu* (Xie et al., 2018).

In cheese ripening, yeasts and some lactic acid bacteria transform amino acids including valine, leucine, isoleucine, phenylalanine, tryptophan, tyrosine and methionine into odor volatiles such as aldehydes, alcohols and carboxylic acids by amino acid catabolism pathway/Ehrlich pathway (Dzialo et al., 2017; Yvon and Rijnen, 2001). The sulphur compounds including methional, methanethiol, dimethyldisulphide and dimethyltrisulphide are also major aroma compounds of cheese, resulting from methionine degradation by cheese microorganisms such as *Brevibacterium lines* and *Geotrichum candidum* through amino acid elimination pathway (Berger et al., 1999; Dias and Weimer, 1998). However, the sulphur compounds are usually absent from fermented soy foods due to the limitation of methionine in the soy protein.

Free fatty acids are either aroma compounds such as butyric acid and hexanoic acid, or important precursors of aroma compounds such as esters, ketones, lactones, and secondary

alcohols (Thierry et al., 2017). Cheese ripening microorganisms including *Propionibacterium freudenreichii*, *Yarrowia lipolytica*, *Geotrichum candidum*, and *Penicillium* spp. have high lipase activities, releasing free fatty acids from fat (Abeijón Mukdsi et al., 2014; Corzo and Revah, 1999; Gaborit et al., 2001; McSweeney and Sousa, 2000). During sufu ripening, a variety of ethyl esters were synthesized from the ethanol in dressing mixtures and fatty acids (Liang et al., 2019; Yao et al., 2021).

The salt content directly influences the overall acceptance of food. In order to inhibit the growth of undesirable microbiota, the high concentrations of salt solution are frequently utilized in the fermentation of *douchi* and *sufu*. The salt contents of final *douchi* and *sufu* products range from 5 to 18%, imparting a very salty taste to these two products. However, the salt content of cheese varies among different-type products, ranging from 0.5 to 6% (Guinee, 2004), which is significantly lower than that in *douchi* or *sufu*. There were more high salt tolerant microorganisms survived in post-fermentation of *douchi* or *sufu*, compared to those in cheese ripening.

## 2.5 Conclusions

Fermented soy foods exhibit the unique characteristics as a result of specific fermentation process and microbial activity. The heating step inactivates the vegetative microorganisms and enzymes in raw soy beans prior to the major fermentation. Thus, with the exception of stinky tofu, fermented soy foods use fungi and *Bacillus* spp. as starter cultures to produce a variety of hydrolytic enzymes to decompose proteins, polysaccharides and lipids at the initial stage of fermentation. There is no addition of salts in the fermentation of *natto* and *tempeh*. Their flavor and quality are highly related to the microbial activity of starter cultures. However, *douchi* and *sufu* undergo a long-time maturation and the addition of salts is essential to control the undesirable microorganism growth during the maturation. The lactic acid bacteria and yeasts dominate at the

end of post-fermentation and finally impart the characteristic flavor and texture to the final products by a series of microbial enzymatic reactions. The understanding of major fermentation microbes and the major pathways for flavor formation in the production of traditionally fermented soy may provide helpful templates for the design of plant-based fermented products.

# Chapter 3. Characterization of γ-glutamyl cysteine ligases from *Limosilactobacillus reuteri* producing kokumi active γ-glutamyl dipeptides

## 3.1 Introduction

γ-Glutamyl peptides are potent bioactives which interact with vertebrate Calcium-sensing proteins (J. Yang et al., 2019). When present in micromolar concentrations, γ-glutamyl peptides modulate signaling cascades that relate to taste perception and inflammation (Ohsu et al., 2010; H. Zhang et al., 2015). *In vivo* anti-inflammatory activity was demonstrated for dietary γ-Glu-Val (H. Zhang et al., 2015; Zhang et al., 2016). γ-Glutamyl-dipeptides were also act as taste enhancers with kokumi taste activity (Toelstede and Hofmann, 2009; Ueda et al., 1994) which is described as "mouthfulness", "continuity" and "thickness" (Beksan et al., 2003; Sforza et al., 2006; Toelstede et al., 2009; Toelstede and Hofmann, 2009). Several γ-glutamyl dipeptides are known to be kokumi-active including γ-Glu-Glu, γ-Glu-Gln, γ-Glu-Gly, γ-Glu-Ala, γ-Glu-Val, γ-Glu-Met, γ-Glu-Leu, γ-Glu-Phe, and γ-Glu-His. The threshold for kokumi activity was reported to range from 5 μmol / kg to 2.5 mmol / kg, however, a standardized protocol for determination of the threshold for kokumi-activity through sensory analyses is not available. Moreover, the kokumi threshold depends on the food matrix in which kokumi-activity is determined (Glabasnia and Hofmann, 2006; Stark and Hofmann, 2005; Toelstede et al., 2009; J. Yang et al., 2019). Kokumi-active γ-glutamyl peptides occur in wheat (Sarwin et al., 1992), edible beans (Liao et al., 2013), garlic (Nakamoto et al., 2018) and onions (Ueda et al., 1994). In addition to their presence in plants, microbial activity generates γ-glutamyl dipeptides in food fermentation including ripened cheese (Toelstede and Hofmann, 2009), soy sauce (Kuroda and Miyamura, 2015) and sourdough for bread-making (Zhao and Gänzle, 2016). Because kokumi-active compounds substantially contribute to the taste of some foods, they are an attractive tool for food product development and

may allow reduction of the salt and sugar content of foods without compromising consumer acceptance (J. Yang et al., 2019; Zhao and Gänzle, 2016).

Microbial enzymes involved in the synthesis of γ-glutamyl dipeptides include γ-glutamyl transferase/transpeptidase (GGT, EC 2.3.2.2, Suzuki et al. 2007), glutaminase (EC 3.5.1.2, Nandakumar et al. 2003) and γ-glutamyl-cysteine ligase (EC 6.3.2.2, Roudot-Algaron et al. 1994). The transpeptidase activity of GGT generates γ-glutamyl dipeptides *in vitro* and food fermentations (Hillmann et al., 2016; Toelstede and Hofmann, 2009). At pH-values higher than 7.5, fungal and bacterial glutaminases catalyze the formation of γ-glutamyl dipeptides from glutamate and amino acids *in vitro* (Tachiki et al., 1996; Tomita et al., 1989; Yang et al., 2017) but glutaminases of lactobacilli do not contribute to formation of kokumi-active compounds in food fermentations (Q. Li et al., 2020). Evidence for the contribution of Gcl is provided by the strain-specific accumulation of γ-glutamyl dipeptides in sourdough fermentation with *Limosilactobacillus reuteri* (Zhao and Gänzle, 2016). The role of two Gcls in the biosynthesis of several γ-glutamyl dipeptides has been confirmed with Gcl-deficient mutant strains of *Lm. reuteri* LTH5448, indicating that *gcl1* and *gcl2* were responsible for γ-Glu-Ile and γ-Glu-Cys synthesis, respectively (Yan et al., 2018).

γ-Glu-Cys ligases (Gcls) are also the rate-limiting enzyme in the synthesis of glutathione (GSH). Gcls synthesize γ-glu-cys in an ATP-dependent reaction by ligating the γ-carboxyl group of glutamate to cysteine (Figure 3.1). Gcls have been characterized from several bacteria (Kelly et al., 2002; Kino et al., 2007; Vergauwen et al., 2006) and yeast (Kong et al., 2018). Gcl domains of GshAB from *Streptococcus agalactiae* and *Escherichia coli* catalyze the biosynthesis of several γ-glutamyl dipeptides (Figure 3.1). Heterofermentative lactobacilli frequently harbor *gcl* genes and genomes of strains in the genera *Lentilactobacillus* and *Limosilactobacillus* frequently encode for

two or three *gcl* genes. In these organisms, the function of Gcls is not related to glutathione synthesis because genes coding for GSH synthetase (GS, EC 6.3.2.3) are generally absent (Pophaly et al., 2012; Yan et al., 2018; Zheng et al., 2015b). However, few studies characterized the activity of Gcl enzymes of food-fermenting lactobacilli to elucidate their contribution to synthesis of γ-glutamyl dipeptides. It was therefore the aim of this study to characterize three Gcls from *Lm. reuteri* and assess their contribution in the synthesis of kokumi-active γ-glutamyl dipeptides.

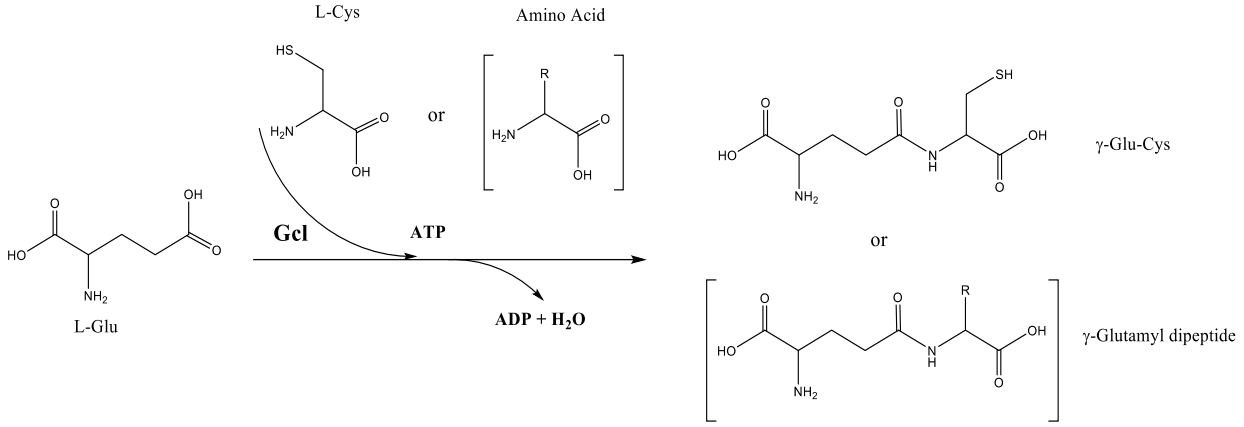



**Figure 3.1.** Enzymatic reactions catalyzed by Gcl for synthesis of γ-glutamyl dipeptides.

## 3.2 Materials and methods

### 3.2.1 Bacteria strains and growth conditions

*Lm. reuteri* subsp. *murium* LTH5448 (NZ_JOOG00000000), which encodes for Gcl1 and Gcl2, and *Lm. reuteri* subsp. *rodentium* LTH2584 (NZ_JOSX00000000), which encodes for Gcl1, Gcl2, and Gcl3, were grown at 37 °C in modified deMan-Rogosa-Sharpe (mMRS) medium with the following ingredients per liter: 5 g beef extract, 5 g yeast extract, 10 g peptone, 10 g malt extract, 10 g maltose, 5 g fructose, 5 g glucose, 2.6 g $KH_2PO_4$, 4 g $K_2HPO_4$, 3 g $NH_4Cl$, 0.5 g cysteine HCl, 1 g Tween 80, 0.1 mg $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot H_2O$. *E.coli* DH5α and *E. coli* BL21 star (DE3), which were used as a host for the construction of plasmids pET-28a(+) with

the insert gene and the over-expression of recombinant proteins, respectively, were grown in Luria-Bertani (LB) medium (BD, Mississauga, CA, U.S.A.) at 37 °C with shaking. Kanamycin (50 mg/L) was added into LB medium for *E. coli* growth carrying plasmids pET-28a(+).

### 3.2.2 Chemicals and reagents

2,3-Naphthalenedicarboxaldehyde (NDA), ATP, 5-sulfosalicylic acid (SSA), $Me_2SO$, L-buthionine-sulfoximine (L-BSO), L-amino acids and other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). γ-Glutamyl dipeptides (γ-Glu-Ala, γ-Glu-Leu, γ-Glu-Ile, γ-Glu-Phe, γ-Glu-Met, γ-Glu-Pro, γ-Glu-Gly, γ-Glu-Ser, γ-Glu-Glu, γ-Glu-Gln, and γ-Glu-Asp) were obtained from United Biosystems (Herndon, VA, U.S.A.).

### 3.2.3 DNA manipulations

The Blood & Tissue Kit (Qiagen, Hilden, Germany) and GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Scientific, Mississauga, CA, U.S.A.) were used for the isolation of genomic DNA and the extraction of plasmid DNA, respectively. Primers were obtained from Integrated DNA Technologies (San Diego, CA, U.S.A.). Phusion High-Fidelity DNA polymerases, T4 DNA ligase and restriction enzymes were obtained from Thermo Scientific. PCR fragments were purified with GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Scientific). Sanger sequencing of PCR products was performed by the Molecular Biology Service Unit (MBSU) of the Department of Biological Sciences at the University of Alberta.

### 3.2.4 Cloning and heterologous expression of Gcl from *Lm. reuteri*

The *gcl* genes (Gcl1, WP_035156810.1; Gcl2, WP_085680095.1; Gcl3, KEK14969) from *Lm. reuteri* LTH5448 and LTH2584 were amplified with primers shown in Table 3.1. The *gcl* PCR fragments and expression vector pET-28a(+) were purified, digested with the same restriction endonucleases (Table 3.1), and ligated into plasmids pET-28a(+)/*gcl* using T4 DNA ligase.

Recombinant plasmids were; transformed into *E. coli* DH5α and sequences of the inserted *gcls* were verified by DNA sequencing. The resulting plasmids pET-28a(+) with the *gcl* genes were extracted from *E. coli* DH5α and introduced into the expression host *E. coli* BL21 star (DE3). *E. coli* BL21 (DE3) strains with pET-28a (+) carrying *gcl1*, *gcl2* or *gcl3* were incubated in LB broth at 37 °C with 180 rpm agitation. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.2 mM) was added to induce the over-expression of recombinant proteins when the optical density (OD) of the culture at 600nm was between 0.4 and 0.6, followed by further incubation for 18 h at 25 °C. Cells were harvested by centrifugation at 4 °C and stored at -80 °C until use.

**Table 3.1** Primers used in this study[a].

| Primer (forward, F; reverse, R) | Sequence (5'-3') | Restriction site |
|---|---|---|
| *gcl1* cloning, F | 5'-ATGCA GGATCCATGTTTAGCAGAATTGG-3' | *Bam*HI |
| *gcl1* cloning, R | 5'-ATGCA CTCGAGCAATGTTAATTCTTTTCG-3' | *Xho*I |
| *gcl2* cloning, F | 5'-ATGCA GGATCCATGGGAACCGATTATGATC-3' | *Bam*HI |
| *gcl2* cloning, R | 5'-ATGCA CTCGAGCTTTTCCTGAAAATCCTG-3' | *Xho*I |
| *gcl3* cloning, F | 5'-ATGCA GGATCCATGTTAAGTAAATTTGGG-3' | *Bam*HI |
| *gcl3* cloning, R | 5'-ATGCA CTCGAGTTTTGCCGATAAATATTGC-3' | *Xho*I |
| *gcl1*, *gcl2* or *gcl3* sequencing, F1 | 5'-TAATACGACTCACTATAGG-3' | - |
| *gcl1* sequencing, R1 | 5'-GTAAAATCACCGGTAAATTTGG-3' | - |
| *gcl1* sequencing, F2 | 5'-CCAAATTTACCGGTGATTTTAC-3' | - |
| *gcl2* sequencing, R1 | 5'-CCATGAAGTGCTTTAGTTCTG-3' | - |
| *gcl2* sequencing, F2 | 5'-CAGAACTAAAGCACTTCATGG-3' | - |
| *gcl3* sequencing, R1 | 5'-CGTGAATTTAGTTCCAAAACCG-3' | - |
| *gcl3* sequencing, F2 | 5'-CGGTTTTGGAACTAAATTCACG-3' | - |
| *gcl1*, *gcl2* or *gcl3* sequencing, R2 | 5'-GCTAGTTATTGCTCAGCGG-3' | - |

[a]Restriction sites are underlined.

### 3.2.5 Purification of Gcl1, Gcl2, and Gcl3 and SDS-PAGE gel analysis

Gcl1 and Gcl2 were over-expressed as soluble proteins while the over-expressed Gcl3 was present in inclusion bodies (data not shown). The purification of soluble Gcl1 or Gcl2 was performed by HisPur Ni-NTA spin column (Thermo Scientific) according to the instructions of the supplier. The proteins were finally eluted into 50 mM Tris-HCl buffer at pH 8.0 and stored in 15% glycerol at -20 °C until use. Inclusion bodies containing Gcl3 inclusion bodies were solubilized and refolded by using the protein refolding kit (Novagen) according to the manufacturer's instruction. Refolded Gcl3 was then concentrated with a 10 KDa centrifugal filter unit (Merck Millipore Ltd., Carrigtwohill, Ireland) and stored in 15% glycerol at -20 °C until use. The enzymatic activity of three Gcls remained stable over 6 months of storage (data not shown).

The protein sample was mixed with 2× Laemmli sample buffer (Bio-Rad, Hercules, California, U.S.A) with 5% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, U.S.A.) (freshly made) in a ratio of 1:1, heated at 95 ºC for 5 minutes, and loaded into the gel wells. SDS-PAGE was performed using a Bio-Rad PowerPac 300 Electrophoresis system (Bio-Rad) with 10% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, California, U.S.A) at 150 V voltage and 400 mA current for 55 min. Precision Plus Protein™ Dual Color Standards (Bio-Rad) served as standards. The gel was stained using Coomassie Blue for visualization of protein bands.

### 3.2.6 Biochemical characterization of Gcls

Biochemical characteristics of the three Gcl enzymes were assayed with a microtitre-plate based fluorescence method to quantify γ-Glu-Cys (White et al., 2003). After precipitating proteins with SSA, the microtitre-plates were kept on ice for 20 min and the precipitate was removed by centrifugation. Aliquots of 20 µl of the reaction supernatant were transferred to a 96-well black plate (Corning Incorporated, Kennebunk, U.S.A) and mixed with 180 µl of NDA derivatization

solution(White et al., 2003). The plate was covered and incubated in dark for 30 min at ambient temperature. The fluorescence intensity of NDA-γ-Glu-Cys or GSH was determined with an excitation wavelength of 472 nm and an emission wavelength of 528 nm with a fluorescence plate reader (Varioskan Flash, Thermo Electron Corporation, CA, USA). A standard curve for quantification of γ-glu-cys was generated with glutathione (GSH).

To assay the inhibition of Gcl by L-buthionine-sulfoximine (L-BSO), 5µl of L-BSO solution was added as a final concentration (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 1.0 mM, or 2.0 mM) into the mixture of 75 µl of reaction cocktail (50 mM Trizma Base, 40 mM ATP, 20 mM Glu, 40 mM MgCl2, and 2 mM EDTA, pH 8.0) with 25 µl of enzyme. The addition of 5 µl of $H_2O$ without L-BSO was used as reference. The reaction mixtures with L-BSO or water were pre-incubated for 5 min at 37 °C, followed by pipetting 50 µl of 5 mM of cysteine solution (pH 8.0) to initiate the enzymatic reaction. The reactions were carried out for 45 min at 37 °C and stopped by 50 µl of 500 mM SSA. In a reaction control, the cysteine solution was not pipetted into the mixture until the addition of SSA.

To determine the effect of metal ions on the Gcl activity, 5µl of metal ion solutions (400 mM of $Mg^{2+}$ or 100 mM of $K^{+}$, $Mn^{2+}$, $Ca^{2+}$, $Zn^{2+}$, $Ba^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Fe^{2+}$, or $Fe^{3+}$) were pipetted into 100 µl of the reaction mixture (pH 8.0) without metal ions. Addition of 5 µl of water served as control. After a pre-incubation for 5 min at 37 °C, 50 µl of 5 mM of cysteine solution (pH 8.0) was used to initiate the reaction. Following an additional incubation for 45 min at 37 °C, 50 µl of 500 mM SSA was added to terminate the reaction. For each metal ion, the corresponding controls were carried out in parallel.

To determine the optimal pH of enzymatic reaction by Gcl, reaction cocktails (40 mM ATP, 20 mM Glu, 40 mM MgCl2, and 2 mM EDTA) were prepared with pH values ranging from 4.0 to

10.5. The following buffers were used: 50 mM of citrate buffer (pH 4.6 0 6.0), 50 mM of Tris-HCl buffer (pH 7.0 – 9.0) and 50 mM of carbonate-bicarbonate buffer (pH 9.5−10.5). Cysteine was dissolved in the buffer corresponding to the pH of reaction cocktail to a final concentration of 5 mM cysteine. The reaction mixtures containing 75 µl of reaction cocktail and 25 µl of Gcl enzyme were pre-warmed for 5 min at 37 °C, then mixed with 50 µl of 5 mM of cysteine solutions to start the reaction, followed by incubation for 45 min at 37 °C. The reaction was stopped by the addition of 50 µl of 500 mM SSA. Reaction controls where cysteine was added only after addition of SSA were conducted for each pH.

To assay the effect of temperature on the activity of Gcl, the enzymatic reaction was carried out at different temperature ranging from 10 to 70 °C. 25 µl of Gcl enzyme was pipetted into the reaction cocktail (50 mM Trizma Base, 40 mM ATP, 20 mM Glu, 40 mM $MgCl_2$, and 2 mM EDTA, pH 9.0), then pre-incubated for 5 min at desirable temperature. Following that, 50 µl of 5 mM of cysteine solution (pH 9.0) was added to start the enzymatic reaction, which was terminated with 50 µl of 500 mM SSA. Reaction controls were performed at each temperature. Results are reported as means ± standard deviation of three biological replicates.

### 3.2.7 Kinetic characteristics of Gcls with ubstrate cysteine and glutamate

The kinetic constants $K_m$ and $V_{max}$ of three Gcl enzymes for the two substrates cysteine and glutamate were measured in this study. To assay the kinetic characteristic of Gcl for substrate cysteine, a series of cysteine solutions (pH 9.0) were added to obtain a final concentration of 0.03, 0.10, 0.17, 0.33, 0.50, 0.67, 1.33, 2.00 or 3.33 mM. The concentration of glutamate was 10 mM. Similarly, kinetic constants of Gcl enzymes for glutamate were studies by addition of 50 µl of glutamate solutions (pH 9.0) to a final concentration of 0.3, 1.0, 2.0, 3.0, 5.0, 10.0, 20.0 or 30.0 mM into the mixtures of 75 µl of reaction cocktail (50 mM Trizma base, 40 mM ATP, 5 mM

cysteine, 40 mM MgCl2, and 2 mM EDTA, pH 9.0) with 25 µl of enzyme. All reactions were performed at 43 °C, pH 9.0 with 45 min incubation, then terminated by using 50 µl of 500 mM SSA. The concentration of γ-Glu-Cys was assayed using fluorescence-based microtiter plate assay. The experimental data of the enzymatic activity was fitted to the Michaelis-Menten equation:

$$V = \frac{V_{max}[S]}{K_m + [S]},$$

where V is the reaction velocity, $V_{max}$ is the maximum reaction velocity, [S] is the substrate concentration and $K_m$ is the Michaelis-Menten constant. The parameters $V_{max}$ and $K_m$ were estimated with the non-linear curve fit tool implemented in SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA, U.S.A). Results are reported as means ± standard deviation of three biological replicates.

### 3.2.8 Qualitative assessment of the substrate specificity of Gcls by LC-MS/MS analysis

To assess the substrate specificity of Gcls, 50 µl of amino acid solution (pH 9.0) containing 20 mM of Ala, Gly, Ser, Leu, Ile, Val, Phe, Met, Cys, Pro, Glu, Gln, Asp or His was added into the reaction mixtures of 75 µl of reaction cocktail (50 mM Trizma base, 40 mM ATP, 60 mM Glu, 40 mM $MgCl_2$, and 2 mM EDTA, pH 9.0) with 25 µl of enzyme. All reactions were carried out at 43 °C for 2 h where the highest yield of dipeptides was obtained with Glu and Cys as substrates (data not shown).

γ-Glutamyl dipeptides were separated using an Agilent 1100 series HPLC unit equipped with a Luna Omega polar C18 column (1.6 µm, 50 × 2.1 mm, Phenomenex, Torrance, CA, U.S.A.) and detected using a Micromass Quattro micro API tandem quadrupole LC-MS/MS system (Waters Corporation, Milford, Massachusetts, U.S.A) with multiple reaction monitoring (MRM) mode. Mobile phase A and B consisted of 0.1 % formic acid in Milli-Q water and 0.1 % formic acid in acetonitrile, respectively. Samples were eluted at a flow of 0.3 ml/min as the following

gradients: 0−6.50 min, 100−88% A; 6.50−6.51 min, 88−25% A; 6.51−8.00 min, 25% A; and followed by a column re-equilibration with 100% A for 8 min at a flow of 0.35 ml/min. Data acquisition was interfaced to the Masslynx v4.1 software (Waters Corporation, Milford, Massachusetts, U.S.A).

### 3.2.9 Phylogenetic analysis and gene clustering

Three putative genes coding for Gcl were identified in *Lm. reuteri* (Yan et al., 2018). Two of these sequences, *gcl1* and *gcl2*, are present in the genome of *Lm. reuteri* LTH5448 while *Lm. reuteri* LTH2584 additionally harbors *gcl3*. The protein sequences of glutamyl cysteine ligases (Gcl) and GSH synthetase (GshAB) in all lactobacilli were retrieved from NCBI database using protein sequences of Gcl1, Gcl2 and Gcl3 as a query sequence. One sequence per protein (Gcl or GshAB) from the same species was selected with a cutoff of 25% average amino acid identity. This relatively low cut-off was chosen to cover the range of pairwise amino acid identities(%) of Gcl domains from *Limosilactobacillus reuteri* (Table 3.2); if similar sequences were present in the same species, the sequence with a lower amino acid identity was removed. A total of 221 protein sequences of Gcl and GshAB were selected, then aligned by Muscle in MEGAX. The phylogenetic analysis with the aligned sequences was carried out by IQ-Tree software (iqtree.cibiv.univie.ac.at) using the maximum likelihood method. LG+F+G4 was found as a best-fit model. The phylogenetic tree was displayed in iTOL.

### 3.2.10 Statistical analysis

Data analysis for the relative activity in the inhibition experiment by L-BSO was performed by one-way analysis of variance (ANOVA) using IBM SPSS statistics 23. *P* value of ≤0.05 with Tukey adjustment for multiple comparisons was considered statistically significant.

## 3.3 Results

### 3.3.1 Expression and purification of glutamyl cysteine ligases from *Lm. reuteri*

To determine whether different Gcls that are present in the same genome of strains of *Lm. reuteri* differ with respect to their biochemical properties, Gcl1, Gcl2 and Gcl3 were purified after heterologous expression in *E. coli*. SDS-PAGE analysis of purified Gcl1 and Gcl2 demonstrated a single band which was absent in the crude cellular extracts of *E. coli* with the empty plasmid pET28a (+) (Figure 3.2). SDS-PAGE analysis of Gcl3, which was purified from inclusion bodies, indicated the presence of the refolded Gcl3 as a major band together with few other faint bands (Figure 3.2). The molecular weights of Gcl1, Gcl2 and Gcl3 as estimated by SDS-PAGE were approximately 5 kDa larger than the predicted molecular weight of 51.10, 59.29 and 49.34 kDa (www.bioinformatics.org/sms/prot_mw.html), respectively.

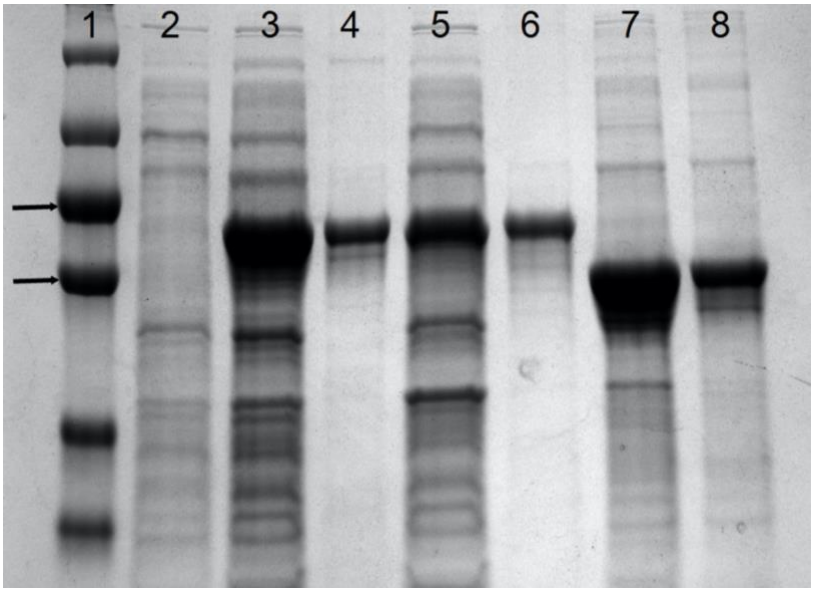



**Figure 3.2.** SDS-PAGE of γ-glutamyl-cysteine ligases (Gcls) over-expressed in recombinant *Escherichia coli* BL21(DE3). Lane 1, molecular weight marker, the upper and lower arrows indicate proteins with 70 and 55kDa, respectively; lane 2: uninduced cell lysate of *E. coli* with pET28a$^{+}$; lane 3: induced cell lysate of *E. coli* with pET28a$^{+}$(*gcl1*); lane 4: purified Gcl1; lane 5: induced cell lysate of *E. coli* with pET28a$^{+}$(*gcl2*); lane 6: purified Gcl2; lane 7: Gcl3 inclusion body and cell wall preparation of induced *E. coli* with pET28a$^{+}$(*gcl3*); lane 8: renatured Gcl3).

The pairwise amino acid identity of Gcl domains from Gcl1, Gcl2 and Gcl3 is shown in Table 3.2. The 337 amino acid Gcl domain of Gcl2 was 31.1% identical to the Gcl domain of Gcl1 (315 amino acids) and 28.0% identical to the Gcl domain of Gcl3 (314 amino acids). The Gcl domains of Gcl1 and Gcl3 were more than 40% identical. *S. agalacticae* harbors *gshAB* encoding two-domain GSH synthetase (Janowiak and Griffith, 2005) and the Gcl domain of GshAB (320 amino acids) was 33−35% identical to the Gcl domains of *Lm. reuteri* enzymes (Table 3.2).

**Table 3.2.** Pairwise amino acid identity (%) of Gcl domains from *Limosilactobacillus reuteri* and *Streptococcus agalacticae* (QDK30862.1)[a)].

| | **Gcl1** | **Gcl2** | **Gcl3** |
|---|---|---|---|
| Gcl1 domain from *Lm. reuteri* | Q[b)] | | |
| Gcl2 domain from *Lm. reuteri* | 31.1 | Q | |
| Gcl3 domain from *Lm. reuteri* | 46.4 | 28.0 | Q |
| Gcl domain from *S. agalacticae* | 34.1 | 35.4 | 33.3 |

[a)] The 320 amino acid Gcl domains of the proteins was identified by InterPro software (www.ebi.ac.uk/interpro) and used as BLAST query on NCBI.
[b)] Q, query sequence with 100% identity.

### 3.3.2 Inhibition of Gcl by L-buthionine-sulfoximine (L-BSO)

L-BSO inhibits Gcl enzymes by binding to the active site of Gcl, which disturbs binding of cysteine (Hibi et al., 2004; Janowiak and Griffith, 2005; Kelly et al., 2002). To measure the inhibition of Gcl1, Gcl2 and Gcl3 by L-BSO, the reactions for these three enzymes were performed in presence of different concentrations of L-BSO. The results indicated that 0.3 mM L-BSO reduced activity of the three Gcls from *Lm. reuteri* by 50% (Figure 3.3). The inhibition of Gcl1 and Gcl2 was similar but activity of Gcl3 was further reduced by 75% if the concentration of L-BSO was increased to 0.6 mM. These results suggest that inhibition of Gcls from *Lm. reuteri* by L-BSO is less pronounced when compared to Gcl from *E. coli* or mammalian Gcls (Griffith, 1982; Kelly et al., 2002).

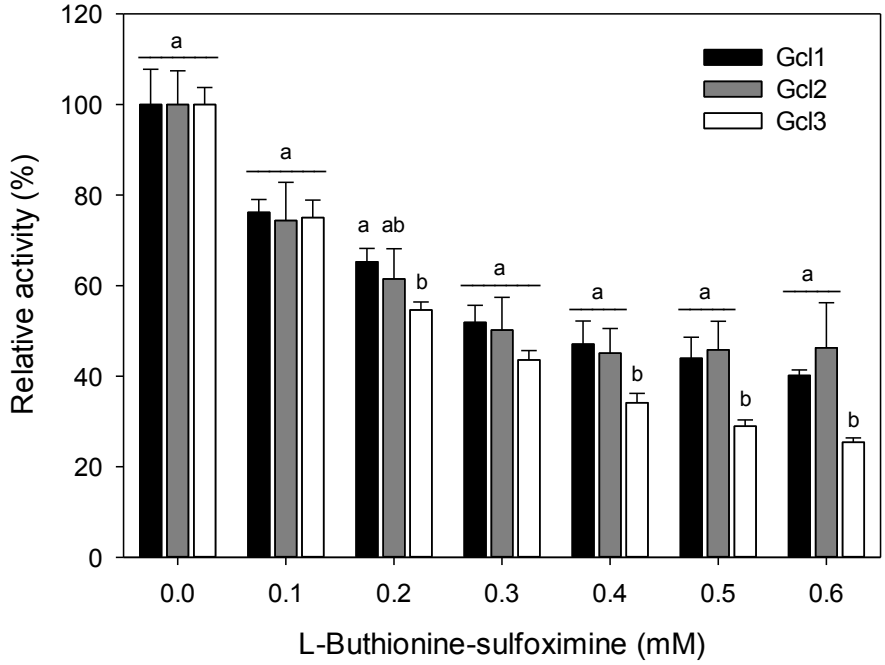



**Figure 3.3.** Relative activity of Gcl1, Gcl1, and Gcl3 in presence of the Gcl inhibitor L-buthionine-sulfoximine (L-BSO). Enzymatic reactions were carried out in presence of 10.0mM Glu and 1.7 mM Cys at pH 8.0 and 37 °C.

### 3.3.3 The Effect of metal ions on the Gcl Activity

In the reaction catalyzed by the ATP-dependent glutamyl cysteine ligase, divalent metal ions play a key role in ATP binding, phosphoryl transfer, the stabilization of the structure of γ-glutamylphosphate intermediate in transition state and the elimination of ADP and phosphate (Abbott et al., 2001; Hibi et al., 2004). Particularly $Mg^{2+}$ and $Mn^{2+}$ are key co-factors for the activity of Gcl enzymes. To assay the role of metal ions in the activity of *Lm. reuteri* Gcls, $Mg^{2+}$, $Mn^{2+}$ and 9 other metal ions were used in enzymatic reactions (Figure 3.4), respectively. Gcls from *Lm. reuteri* Gcls were inactive unless in the absence of $Mg^{2+}$ or $Mn^{2+}$. Other metal ions, including $K^{+}$, $Ca^{2+}$, $Zn^{2+}$, $Ba^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Fe^{2+}$ and $Fe^{3+}$, did not support activity of Gcls of *Lm. reuteri*. The activity of Gcls in presence of $Mg^{2+}$ was substantially higher than the activity in presence of

$Mn^{2+}$, however, the concentration of $Mn^{2+}$ was 4-fold lower than that of $Mg^{2+}$ because higher concentrations of $Mn^{2+}$ precipitated all three Gcl proteins (data not shown).

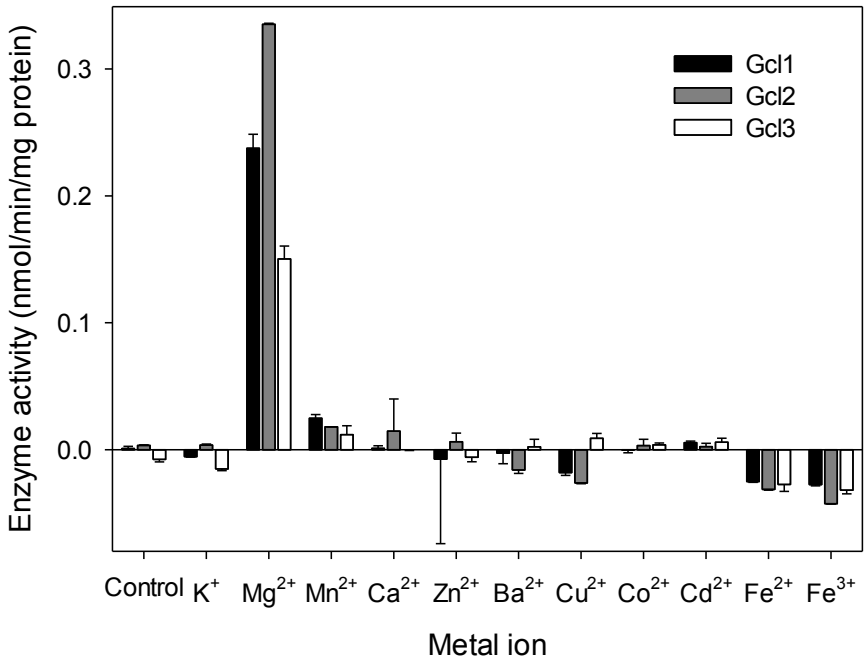



**Figure 3.4.** Activity of Gcl1, Gcl2 and Gcl3 in presence of 13.3 mM of $Mg^{2+}$ or 3.3 mM of all other metal ions. Enzymatic reactions were carried out in presence of 10.0 mM Glu and 1.7 mM Cys at pH 8.0 and 37 °C.

### 3.3.4 Determination of the optimal pH and temperature of the enzymatic reaction by Gcls

Gcl1, Gcl2 and Gcl3 were characterized with regards to their activity at pH 4.0 – 10.5 and in the temperature range of 10 – 70 ° (Figure 3.5). All three *Lm. reuteri* Gcl enzymes were optimally active at pH 9.0; the minimal pH was 6.0 for all three enzymes. Gcl3 was less active than Gcl1 and Gcl2 at pH values higher than pH 9.0. The activities of Gcl1 and Gcl2 were also reduced at a pH of 10.5. Gcl1, Gcl2 and Gcl3 showed a similar response to the reaction temperature. The optimal temperature of all three Gcl enzymes was 50 °C, and 90% relative activity was

achieved when performing the reaction at 43−60 °C. Gcl1, Gcl2 and Gcl3 remained active at 10 °C but were completely inactivated at 70 °C.

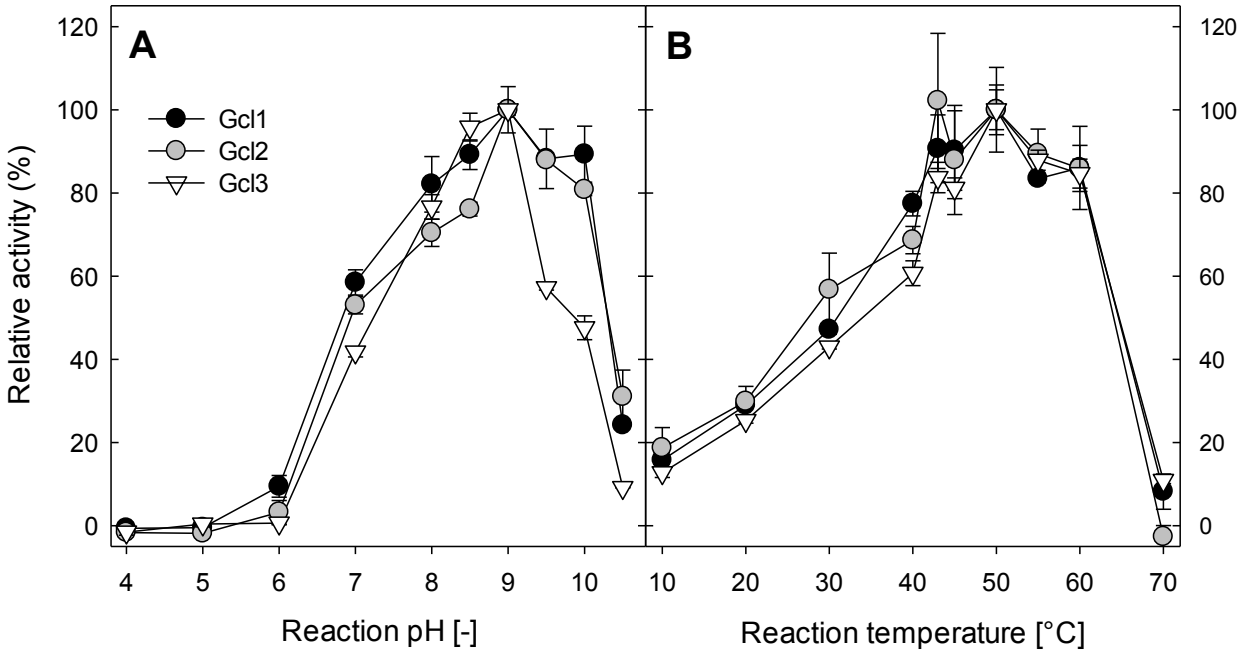



**Figure 3.5.** Relative activity of Gcl1, Gcl2 and Gcl3 at different pH values and at different temperatures. Enzymatic reactions were carried out in presence of 10.0 mM Glu and 1.7 mM Cys; the effect of the pH was assayed at a temperature of 37 °C; the effect of the temperature was assayed at pH 9.0.

### 3.3.5 Kinetic characteristics of Gcls with substrate cysteine and glutamate

Cysteine and glutamate are the substrates for synthesis of γ-Glu-Cys by Gcls. For determination of the $K_m$ and $V_{max}$ values of Gcls for Cys or Glu, other substrates and co-factors for Gcl activity were added to saturation. The concentration of γ-Glu-Cys synthesized by Gcls linearly increased for the first 60 min of the reaction (data not shown). A 45 min incubation time was thus utilized to determine the kinetic constants for Glu and Cys. The experimental data was fitted to the Michaelis-Menten kinetics and difference between experimental and predicted values

was smaller than the experimental error for most data points (Figure 3.6). The $K_m$ values for Gcl1, Gcl2 and Gcl3 with substrate Cys were 0.11, 0.10 and 0.18 mM, respectively, and the $K_m$–values for the substrate Glu were 1.54, 1.46 and 1.56 mM, respectively. All three enzymes thus exhibited a 10-fold higher affinity for Cys than for Glu. Gcl3 exhibited higher $V_{max}$ values with Cys and Glu than Gcl1 and Gcl2.

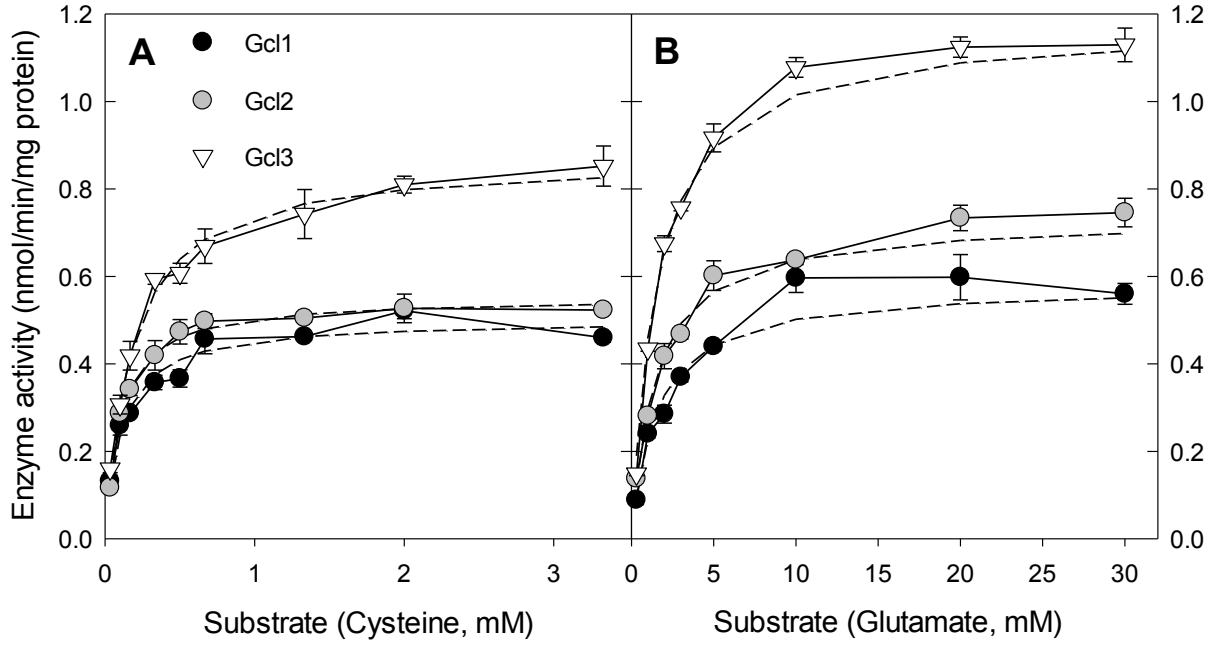



**Figure 3.6.** Kinetic characteristics of Gcl1, Gcl2 and Gcl3 with cysteine and glutamate as substrates. Enzymatic reactions were carried out at pH 9.0 and 43 °C and 10.0 mM Glu (Panel A) and 1.7 mM Cys (Panel B).

### 3.3.6 Substrate specificity of *Lm. reuteri* Gcls

To evaluate the activity of Gcls with amino acids other than cysteine as second substrate, the reaction was performed with glutamate and one each of 14 amino acids (Table 3.3). The amino acids were chosen to include the substrates for 10 γ-glutamyl dipeptides with demonstrated kokumi activity (Zhao et al., 2016), and to additionally include polar and charged amino acids. Of the amino acids tested, Ala, Met, Cys, Glu and Gln were good acceptors for all three Gcls. None of

the three enzymes was active with Asp, Val and His. Gcl3 exhibited a broader substrate specificity compared to Gcl1 and Gcl2 and produced γ-glutamyl dipeptides with Gly, Ser and Pro, which were not observed in reactions with Gcl1 and Gcl2. Gcl2 differed from Gcl1 and Gcl3 as the hydrophobic amino acid Leu, Ile and Phe were not used as substrates to form the corresponding γ-glutamyl dipeptides.

**Table 3.3.** Qualitative analysis of γ-glutamyl peptides produced by Gcl1, Gcl2, or Gcl3 by LC-MS/MS.

| γ-glutamyl dipeptides | Ion transition (m/z) | Retention time | Gcl1 | Gcl2 | Gcl3 |
|---|---|---|---|---|---|
| γ-glutamyl glutamate | 277.2/148.1 | 0.82 | + | + | + |
| γ-glutamyl glutamine | 276.2/147.1 | 0.95 | + | + | + |
| γ-glutamyl methionine | 279.2/150.1 | 0.95 | + | + | + |
| γ-glutamyl cysteine | [a)]308.2/179.1 | 0.80 | + | + | + |
| γ-glutamyl alanine | 219.2/90.1 | 0.82 | + | + | + |
| γ-glutamyl glycine | 205.1/76.05 | 0.77 | - | - | + |
| γ-glutamyl serine | 235.2/106.1 | 0.75 | ±[b)] | - | + |
| γ-glutamyl leucine | 261.2/132.1 | 6.66 | + | - | + |
| γ-glutamyl isoleucine | 261.2/132.1 | 6.14 | + | - | + |
| γ-glutamyl phenylalanine | 295.3/166.2 | 7.80 | + | - | + |
| γ-glutamyl valine | 247.2/118.1 | n.d.[c)] | - | - | - |
| γ-glutamyl proline | 245.2/116.2 | 0.95 | ±[b)] | - | + |
| γ-glutamyl aspartate | 263.2/148.1 | 0.95 | ±[b)] | - | ± |
| γ-glutamyl histidine | 285.2/156.1 | n.d.[c)] | - | - | - |

[a)] γ-glutamyl cysteine was quantified after derivatization with iodoacetamide
[b)] The signal was less than 10 standard deviations higher than the baseline.
[c)] not detected, i.e. the signal was less than 3 standard deviations higher than the baseline.

### 3.3.7 Phylogenetic analysis of glutamyl cysteine ligases and two-domain GSH synthetases in lactobacilli

To clarify the phylogenetic relationships of three putative *Lm. reuteri* Gcls and all other Gcls and GshABs in lactobacilli (J. Zheng et al., 2020), Gcl and GshAB sequences of lactobacilli were aligned to construct a phylogenetic tree (Figure 3.7). The tree displays four clusters that comprise sequences of Gcl1 and Gcl3, a family of sequences that consisted of GshAB and related Gcls, Gcl2 sequences, and additional Gcl sequences that were mainly present in the genus *Levilactobacillus* and *Lentilactobacillus*. Most of Gcl sequences were present in heterofermentative lactobacilli while most GshAB sequences were present in homofermentative lactobacilli. GshAB sequences clustered separately from Gcl sequences. Gcl2 sequences were mainly identified in strains of the genus *Limosilactobacillus* and clustered separately from Gcl1 and Gcl3. The four Gcl3 sequences from strains in the genus *Limosilactobacillus* clustered separately from Gcl1 sequences in the genera *Limosilactobacillus, Ligilactobacillus* and *Lentilactobacillus* (Figure 3.7) but the phylogenetic tree supports the close relationship of Gcl1 and Gcl3 that is also suggested by the pairwise amino acid identity (Table 3.2).

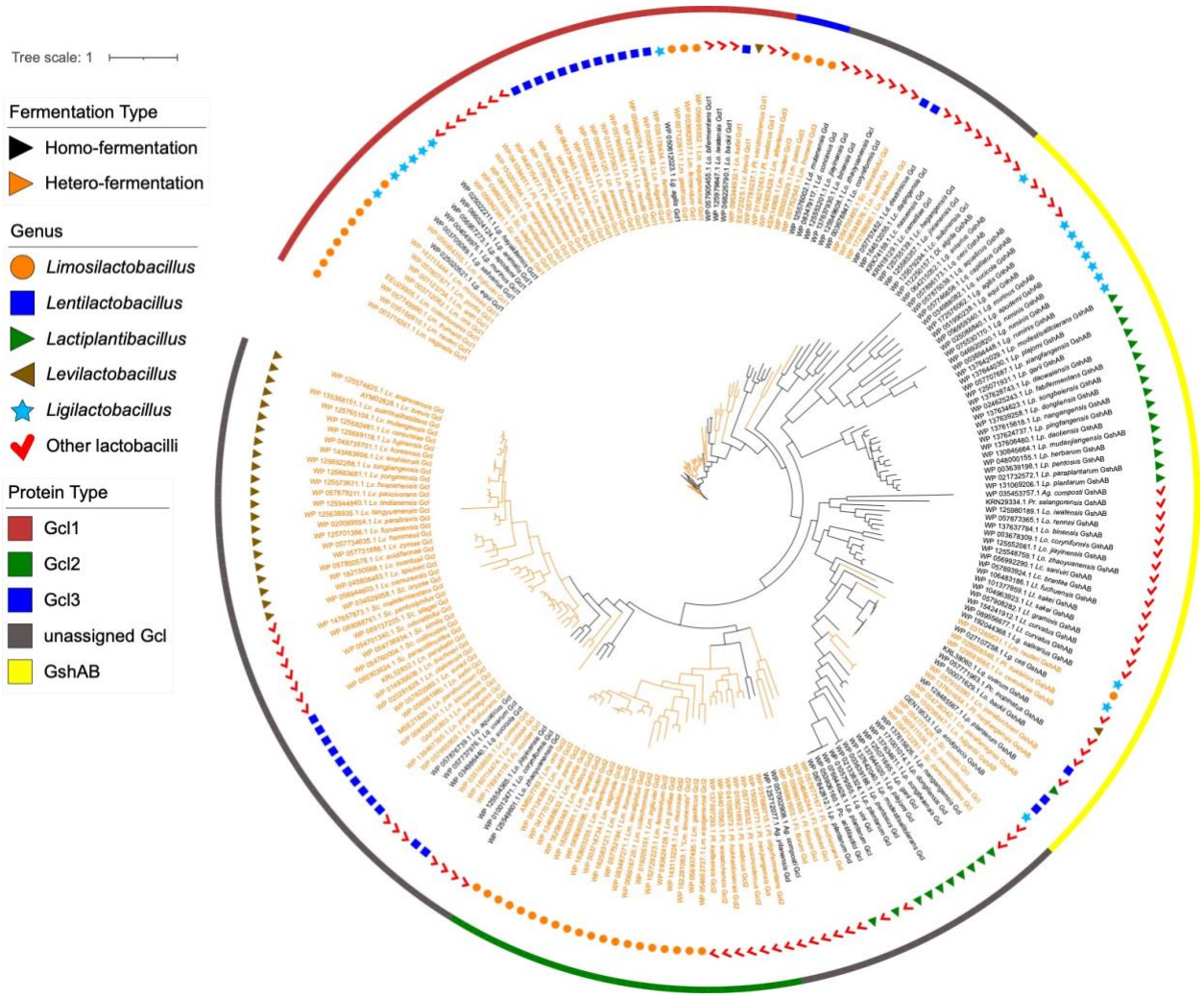



**Figure 3.7.** Phylogenetic tree of γ-glutamyl cysteine ligases (Gcl) and the two-domain GSH synthetase (GshAB) from lactobacilli. NCBI database was searched with Gcl1, Gcl2 or Gcl3 as query sequences with a BLAST cutoff of 25% amino acid identity. The results obtained with the three query sequences were combined and duplicate entries were removed; for each of Gcl1, Gcl2 or Gcl3, one sequence per protein (Gcl or GshAB**)** from the same species was kept to obtain a total of 221 protein sequences**.** Sequences that were more than 40% identical to Gcl1, Gcl2, or Gcl3 were annotated as Gcl1, Gcl2 or Gcl3, respectively; proteins sequences that included the two domains of Gsh synthetases were designated as GshAB; other proteins were designated as Gcl. Lactobacilli and their proteins are color coded as follows: Homofermentative and heterofermentative lactobacilli are coloured in black and orange, respectively. The five most frequent genera of lactobacilli are indicated by symbols; other genera are designated as "other lactobacilli". The protein type (Gcl1, Gcl2, Gcl3, other Gcl, or GshAB) is color coded on the outermost ring.

## 3.4 Discussion

γ-Glutamyl dipeptides improve food quality as kokumi-active compounds (Toelstede et al., 2009; Zhao and Gänzle, 2016) and additionally relieve gut inflammation (H. Zhang et al., 2015; Zhang et al., 2016). This study charracterized three Gcl enzymes from *Lm. reuteri* that synthezise γ-glutamyl dipeptides.

All three Gcls of *Lm. reuteri* were inhibited by L-BSO, as was previously reported for Gcl enzymes (Janowiak and Griffith, 2005; Kelly et al., 2002). The inhibition efficiency of L-BSO to three *Lm. reuteri* Gcls was similar to Gcl domain of GshAB from *S. agalacticae* (Janowiak and Griffith, 2005) but L-BSO was less inhibitory to *Lm. reuteri* Gcls and *S. agalacticae* Gcl when compared to Gcls from *E. coli* or eukaryotes (Kelly et al., 2002). The reduced inhibition by L-BSO has been related to a lower binding affinity of L-BSO to Gcls (Janowiak and Griffith, 2005). Overall, the results for three the Gcls of *Lm. reuteri* and *S. agalacticae* indicate that L-BSO is less inhibitory for Gcls of *Lactobacillales* when compared to Gcl enzymes from other bacteria.

Gcl as an ATP-dependent ligase requires a divalent metal ion for catalysis. Divalent metal ions not only serve as an essential co-factor to activate Gcl but also change its substrate specificity (Kelly et al., 2002; Orlowski and Meister, 1971). Addition of $Mg^{2+}$ or $Mn^{2+}$ was an absolute requirement for the activity of Gcls in *Lm. reuteri*, in keeping with the effect of these ions on activity of other Gcls (Abbott et al., 2001; Janowiak and Griffith, 2005). Gcls of *Lm. reuteri*, however, were not active in presence of $Co^{2+}$, $Cd^{2+}$ and $Fe^{2+}$ while these metal ions activated Gcl of *E. coli* (Kelly et al., 2002).

Although the activity of Gcl has typically been assayed at pH 8.0 − 8.5 and 37 °C (Abbott et al., 2001; Kelly et al., 2002; Kino et al., 2007), the optimal pH and temperature of the three *Lm. reuteri* Gcls were 9.0 and 50 °C. The synthesis of γ-glutamyl dipeptides by Gcl involves a

transpeptidation reaction where the donor γ-glutamyl moiety is transferred to an acceptor nucleophile, i.e. the second amino acid. With regards to the optimum pH of the transpeptidation activity, Gcls are similar to GGT and glutaminase that catalyze γ-glutamyl transfer at alkaline pH in the range of 7.5 − 11 (Morelli et al., 2014; Nandakumar et al., 2003; Suzuki et al., 2007; Tachiki et al., 1998).

All three *Lm. reuteri* Gcls exhibited a higher affinity for cysteine than glutamate, consistent with properties of Gcls in humans (Thulin and Linse, 1998), *E. coli* (Kelly et al., 2002), *S. agalacticae* (Janowiak and Griffith, 2005) and *Pseudoalteromonas haloplanktis* (Albino et al., 2014). The high affinity of Gcls for cysteine may be related to intercellular levels of cysteine. *In vivo* concentrations of cysteine are maintained at relatively low levels (Lee et al., 2004; Stipanuk, 2004). The $K_m$ values of *Lm. reuteri* Gcls for glutamate were similar to each other and to those of human or *E. coli* but 10-fold lower than those of *S. agalacticae* (Janowiak and Griffith, 2005).

The substrate specificity of Gcl or GshAB was previously determined with enzymes from *E. coli* or *S. agalacticae*. These previously characterized Gcl or GshAB exhibited different substrate specificities (Kino et al., 2007). The present study provides an unprecedented characterization of Gcl activity in food fermenting bacteria. Although three *Lm. reuteri* Gcls showed overlapping substrate specificities, Gcl3 had the broadest substrate spectrum among three enzymes. Met and Cys were good acceptors for Gcl1, Gcl2 and Gcl3. Met was also a good γ-glutamyl acceptor for *S. agalacticae* GshAB (Kino et al., 2007) and GGT from *Penicillium roqueforti* (Toelstede and Hofmann, 2009) but not for Glc from *E. coli* (Kino et al., 2007). Similar to Gcl from *E. coli* and GGT, Gcl from *Lm. reuteri* was not active with histidine (Toelstede and Hofmann, 2009). In contrast, γ-Glu-His was synthesized by *S. agalacticae* GshAB (Kino et al., 2007). Gcl1 and Gcl3 were active with the hydrophobic amino acid Leu, Ile and Phe but Gcl2 was

not. Differences of the substrate-specificity between Gcl2 and Gcl1 or Gcl3 relate to their phylogenetic relationship and low amino acid identities, with Gcl2 being most distant from Gcl1 and Gcl3. Gcl3 and Gcl1 are 43% identical but they differed in their substrate specificity, indicating that the specificity of uncharacterized Gcls in *Levilactobacillus* and *Lentilactobacillus* species may also differ.

The distribution of *gcl* or *gshAB* in lactobacilli indicates that Gcl has a different physiological role in heterofermentative and homofermentative lactobacilli. Homofermentative organisms harbor predominantly GshAB, indicating that Gcl acts as part of the GSH biosynthetic pathway to protect against oxidative stress (Fu et al., 2006; Li et al., 2011). In heterofermentative lactobacilli, GSH also protects against environmental stress including oxidative and cold stress (Jänsch et al., 2007; J. Zhang et al., 2010) but most heterofermentative lactobacilli lack the complete pathway for glutathione synthesis (Pophaly et al., 2012). The absence of GSH synthetase activity of GshAB in heterofermentative lactobacilli is compensated by transport and reduction of GSH or GSSG (Jänsch et al., 2007; Pophaly et al., 2012) or by uptake of cysteine and / or methionine (Lo et al., 2009; Stetina et al., 2014). The activity of Gcls in *Lm reuteri* appeared to be unrelated to the resistance to oxidative stress (Yan et al., 2018) and the role of multiple Gcls in the ecology of heterofermentative lactobacilli remains to be investigated. Up to three Gcls that differ in their substrate specificity are present in genomes of *Lentilactobacillus* and *Limosilactobacillus* species. Of the strains used in this study, *Lm. reuteri* LTH5448 encodes for two Gcls and *Lm. reuteri* LTH2584 encodes for all three Gcls. Gcls are absent, however, in other heterofermentative lactobacilli, e.g. *Furfurilactobacillus*, *Apilactobacillus* and *Fructilactobacillus*, which implies that these enzymes contribute to the ecological fitness of lactobacilli only in specific habitats (Duar et al., 2017).

*Lm. reuteri* accumulates kokumi peptides during sourdough fermentation to concentration that exceeds the threshold for kokumi activity; however, their concentration in sourdough bread remains to be determined (Yan et al., 2018; Zhao and Gänzle, 2016). The synthesis of γ-glutamyl dipeptides and their contribution to food flavor differs substantially from the contribution of α-glutamyl dipeptides. α-Glu-dipeptides are released by proteolysis and their taste is dependent on the hydrophobicity of the second amino acid. α-Glutamyl dipeptides with residues of Asp, Glu or Ser possess umami taste while α-Glu-Leu, α-Glu-Ile and α-Glu-Phe taste bitter (Zhao et al., 2016). In contrast, γ-Glu-Leu, γ-Glu-Ile and γ-Glu-Phe are kokumi-active. The present study on Gcl enzymes in lactobacilli extends prior knowledge on their accumulation in food fermentations by an initial characterization of the substrate specificity of three distinct Gcl enzymes. These presence and activity of these enzymes explains the strain specific differences observed in *Lm. reuteri* (Zhao and Gänzle, 2016), however, the expression of the two or three enzymes present in a single strain remains to be determined. All three *Lm. reuteri* Gcls produced kokumi-active γ-Glu-Glu, γ-Glu-Met and γ-Glu-Ala; Gcl1 and Gcl3 but not Gcl2 produced γ-Glu-Leu, γ-Glu-Ile and γ-Glu-Phe and only Gcl3 also produced γ-Glu-Gly, γ-Glu-Pro and γ-Glu-Ser. Of note, the pH during sourdough fermentation, pH 3.5 – 4.0, does not match the optimum pH of Gcl activity, however, the use of *gcl1*- and *gcl2*- deficient mutants of *Lm. reuteri* LTH5448 confirmed their activity during extended fermentation of type II sourdoughs (Yan et al., 2018). Because Gcl1 and Gcl3 produce a wider range of γ-glutamyl dipeptides when compared to Gcl1, the selection of fermentation cultures based on the substrate specificity of their Gcl enzymes may be a suitable tool to accumulate specific γ-glutamyl dipeptides and to improve food flavor through enhanced kokumi activity.

γ-Glu-Val is a potent bioactive that reduces intestinal inflammation (Zhang et al., 2016). None of three Gcls that were characterized in the present study synthesized γ-Glu-Val, however,

other hydrophobic amino acids with unknown *in vivo* anti-inflammatory activity were substrates for Gcl1 and Gcl3. Whether or not *gcl* genes of *Lm. reuteri* regulate inflammatory processes in intestinal tract, or whether these relate to host-adaptation of specific sub-species of *Lm. reuteri* (Frese et al., 2011) thus remains unknown.

## 3.5 Conclusion

The present study characterized three γ-glutamyl cysteine ligases from *Lm. reuteri* that produce kokumi-active γ-glutamyl dipeptides. The differential presence of Gcls and GslAB in homofermentative and heterofermentative lactobacilli suggests that the synthesis of glutathione and γ-glutamyl dipeptides has a different contribution to the ecological fitness of these organisms. Gcl1, Gcl2 and Gcl3 from *Lm. reuteri* were similar with respect to their biochemical properties but differed with respect to their substrate specificities. These different substrate specificities may allow the selection of specific starter cultures for controlled formation of γ-glutamyl dipeptides in food. However, the cytoplasmic concentration of amino acids during growth of lactobacilli in food fermentations differs from the concentration of amino acids in enzymatic reactions that were conducted in the present study, therefore, the contribution of the different Gcls to the synthesis of specific γ-glutamyl dipeptides in food remains to be elucidated.

# Chapter 4. Contribution of γ-glutamyl-cysteine ligases from *Limosilactobacillus reuteri* to the formation of kokumi taste-active γ-glutamyl dipeptides in sourdough or sourdough bread

## 4.1 Introduction

Taste is an important determinant of food quality. Taste is determined by the six basic tastes, sweet, sour, bitter, salty, umami, and oleogustus, which are recognized by oral taste receptors (Running et al., 2015). In addition, the taste perception is determined by kokumi active compounds. Kokumi activity is described as "mouthfulness", "thickness", and "continuity"(Nishimura et al., 2019) and is mediated through the interaction with Calcium-sensing receptor (CaSR) in taste cells that is involved in the signal transduction from taste receptors to the brain (Ohsu et al., 2010). Several peptides with an N-terminal γ-L-glutamyl residue are known to be kokumi active. Kokumi γ-glutamyl peptides in food include γ-glutamyl-S-allyl-L-cysteine, glutathione (γ-Glu-Cys-Gly), γ-Glu-Val-Gly, γ-Glu-Cys, γ-Glu-Glu, γ-Glu-Phe, γ-Glu-Gly, γ-Glu-His, γ-Glu-Leu, γ-Glu-Ile, γ-Glu-Met, γ-Glu-Gln, γ-Glu-Val, γ-Glu-Tyr, and γ-Glu-Ala. These peptides occur in many foods, including galic (Ueda et al., 1990), wheat (Sarwin et al., 1992), onion (Ueda et al., 1994), edible beans (Dunkel et al., 2007), yeast extract (Liu et al., 2015), soy sauce (Kuroda et al., 2013), ripened cheese (Toelstede et al., 2009), or sourdough bread (Zhao and Gänzle, 2016).

While some γ-glutamyl peptides, particularly glutathione, are present in plant foods, the concentration of γ-glutamyl peptides can be increased by microbial or enzymatic conversions during food processing and particularly during food fermentations. γ-Glutamyl transpeptidase activity in garlic and raw milk contributes to the formation of γ-glutamyl-γ-glutamyl-S-allyl-L-

cysteine and γ-glutamyl dipeptide in garlic and cheese (Hillmann et al., 2016; Nakamoto et al., 2018), respectively. γ-Glutamyl dipeptide formation in blue mold cheeses is attributed to γ-glutamyl transpeptidase activity of *P. roquefortii* (Toelstede and Hofmann, 2009). Glutaminases of bacilli, which occur in many alkaline fermented protein foods (Li et al., 2023b), exhibit γ-glutamyl-transferase activity at high pH (Yang et al., 2017). Formation of several kokumi-active γ-glutamyl dipeptides in sourdough is related to the activity of γ-glutamyl cysteine ligases of *Lm. reuteri* (Yan et al., 2018) and the use of sourdough with glutamate and kokumi γ-glutamyl dipeptides increases the taste intensity of bread (Zhao and Gänzle, 2016; Zhao et al., 2015). Moreover, different strains of *Lm. reuteri* differ with respect to the formation of γ-glutamyl dipeptides (Zhao and Gänzle, 2016).

Strains of *Lm. reuteri* harbor up to three *gcl* genes encoding for γ-glutamyl cysteine ligases, designated as Gcl1, Gcl2 and Gcl3 (Chapter 3). These three Gcls exhibit different substrate specificities to acceptor amino acids in the synthesis of γ-glutamyl dipeptides (Chapter 3). The use of Gcl k.o. mutants of *Lm. reuteri* LTH5448 attributes the synthesis of γ-Glu-Ile and γ-Glu-Cys in buffer fermentations to Gcl1 and Gcl2 (Yan et al., 2018), respectively. However, the role of Gcls from *Lm. reuteri* in the accumulation of γ-glutamyl dipeptides in sourdough or sourdough bread remains unknown. The kokumi activity of γ-glutamyl peptide is highly related to its structure associated with the CaSR activity; γ-Glu-Val-Gly showed 30 times higher CaSR activity than glutathione (Ohsu et al., 2010). The kokumi threshold of γ-glutamyl peptides also depends on food matrix (Zhao et al., 2016). The kokumi activity of γ-glutamyl dipeptides in sourdough bread remains to be investigated. It was therefore the aim of this study to investigate the contribution of Gcls from *Lm. reuteri* strains to the synthesis of γ-glutamyl dipeptide in sourdough or sourdough bread and evaluate the kokumi activity of γ-glutamyl dipeptides in bread.

## 4.2 Materials and methods

### 4.2.1 Strains and growth conditions

*Lm. reuteri* subsp. *rodentium* LTH2584, *Lm. reuteri* subsp. *murium* LTH5448, LTH5448Δ*gcl1* and LTH5448 Δ*gcl1*Δ*gcl2* (Yan et al., 2018) were grown in modified deMan-Rogosa-Sharpe (mMRS) medium at 37 °C. Single colonies were inoculated into 10 ml of mMRS broth, followed by an overnight incubation at 37 °C; subculture was obtained by 1% inoculum in broth with an overnight incubation at 37 °C. Cells harvested from the overnight subculture were washed twice using sterile tap water, then resuspended in 10 ml of sterile tap water to be used as inocula for fermentation.

### 4.2.2 Materials and chemicals

Whole wheat flour, all-purpose wheat flour, vital wheat gluten, dextrose, dry baker's yeast and baking soda were obtained in a local supermarket. Food-grade wheat starch, skim milk powder, monosodium glutamate, fructose, L-glutathione, and lactic acid (88% solution) were purchased from Amazon (www.amazon.ca). Fungal protease from *Aspergillus oryzae* was purchased from Sigma-Aldrich (St. Louis, MO, USA). Standards of γ-glutamyl dipeptides (γ-Glu-Ile, γ-Glu-Leu, γ-Glu-Phe, γ-Glu-Cys, γ-Glu-Ala, γ-Glu-Met, γ-Glu-Pro, γ-Glu-Gly, γ-Glu-Ser, γ-Glu-Glu, γ-Glu-Gln and γ-Glu-Asp) were obtained from United Biosystems (Herndon, VA, U.S.A.). Dithiothreitol (DTT), iodoacetamide (IAM), 5-sulfosalicylic acid (SSA), L-amino acids and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

### 4.2.3 Sourdough fermentation with strains of *Lm. reuteri*

To determine the role of *gcl* of *Lm. reuteri* in the formation of γ-glutamyl dipeptides during sourdough fermentation, sourdoughs were prepared with *Lm. reuteri* LTH2584, LTH5448, LTH5448Δ*gcl1*, or LTH5448Δ*gcl1*Δ*gcl2*. The genome of *Lm. reuteri* LTH2584 encodes for Gcl1,

Gcl2 and Gcl3; the genome of *Lm. reuteri* LTH5448 encodes for Gcl1 and Gcl2 and the k.o. mutants encode for Gcl2 only, or are lack all three Gcls (Chapter 3). Inocula of the three strains were prepared by harvesting of cells from 10 mL overnight culture in mMRS and re-suspension in sterile tap water. Five g of whole wheat flour were mixed with 5 g of vital wheat gluten, 0.5 g of fungal protease and 10 mL of the respective inocula to obtain an initial cell count of $10^7$ to $10^8$ cfu/g. Chemically acidified dough with a final pH of 3.5 ± 0.1 by addition of acetic acid/lactic acid (1:4, v/v) served as control. Sourdoughs were incubated at 37 °C and samples were collected after 0, 24, 48, 72 and 96 h of fermentation. Cell counts and pH were determined with fresh sourdoughs. Freeze-dried sourdoughs were used to quantify γ-glutamyl dipeptides. γ-Glutamyl dipeptides were extracted using 0.5 g of dried sourdough with 10 ml of Milli-Q water and stored at – 20 °C prior to use. All sourdough fermentations were performed with three biological replicates. Results are reported as means ± standard deviation.

### 4.2.4 Bread baking

Sourdoughs were prepared with 50 g of whole wheat flour and 50 ml of cultures of *Lm. reuteri* LTH2584 or LTH5448Δ*gcl1*Δ*gcl2* and incubated 24 h at 37 °C. Sourdoughs were further propagated by an addition of 75 g of whole wheat flour, 75 g of vital wheat gluten, 0.75 g of fungal protease, and 350 ml of sterile tap water, followed by incubation at 37 °C for 72 h. After fermentation, sourdoughs were freeze-dried and stored at – 20 °C until baking.

Bread dough preparation and bread baking were similar as described previously (Zhao and Gänzle, 2016). Bread dough was prepared with the following ingredients per 200 g of flour: 180 g of whole wheat flour, 20 g of dry sourdough fermented by *Lm. reuteri* LTH2584 or LTH5448Δ*gcl1*Δ*gcl2* for 72 h, 4 g of sugar, 4 g of yeast, 4 g of salt, and 120 g of water. Doughs were mixed using a spiral kneader (Kitchen Aid K45SS, Hobart Co. Troy, OH, U.S.A.) for 8 min.

Straight dough was prepared by mixing 200 g of whole wheat flour, 4 g of sugar, 4 g of yeast, 4 g of salt, and 120 g of water with a spiral kneader for 8 min. Each of bread doughs was evenly split into three parts, covered with the plastic wrap to reduce water loss from bread dough, and placed in a proofer (Res-Cor, Crescent Metal Products Inc., Cleveland, OH, U.S.A.) with a rest for 2 h at 30 °C. After a dough rest, they were shaped by hand and proofed for an additional hour at 30 °C. Bread was baked in a multideck oven (Bakers Pride, Lachine, QC, Canada) with forced air at a temperature of 182 °C for 20 min. The bread was cooled down at room temperature and cut into pieces. Samples of bread dough and the bread crumb were freeze-dried for the assay of γ-glutamyl dipeptides after proofing. γ-Glutamyl dipeptides were extracted from 0.7 – 1.0 g of dried bread dough or bread with 10 ml of Milli-Q water and stored at – 20 °C prior to analysis.

### 4.2.5 RNA isolation and cDNA library construction of *Lm. reuteri* strains in mMRS and sourdoughs

To determine the expression of *gcl* genes from *Lm. reuteri* strains in mMRS medium during growth, subcultures of *Lm. reuteri* LTH2584, LTH5448, or LTH5448Δ*gcl1* were inoculated into 1 ml of mMRS broth with 1% inoculum and incubated at 37 °C for 3 – 4 h when cultures were grown to exponential phase ($OD_{600nm}$ of 0.6). Cells were harvested by centrifugation at 5000 x g for 10 min; the supernatant was discarded. Cell pellets were resuspended in 1 ml of fresh mMRS medium and mixed with 2 ml of RNA protect bacteria reagent (Qiagen, Mississauga, ON, Canada). After an incubation for 10 min at room temperature, cell pellets were harvested by centrifugation at 5000 x g for 10 min and stored at – 80 °C for less than 2 weeks for RNA isolation.

To quantify the expression of *gcl* from *Lm. reuteri* strains in sourdoughs, sourdoughs were fermented as described above. Samples were collected after 6, 24 and 48 h of fermentation. Sourdough (0.7 – 0.8 g) was homogenized with 10 ml of mMRS broth and centrifuged at 500 x g

for 15 min to remove insoluble dough materials. The supernatant was transferred into a clean tube and bacterial cells were harvested by centrifugation at 5000 x g for 10 min. One ml of broth was used to resuspend cell pellets; 2 ml of RNA protect bacteria reagent was pipetted into 1 ml of cell suspension and incubated at room temperature for 10 min to maintain RNA integrity. Cell pellets were harvested by centrifugation at 5000 x g for 10 min and stored at – 80 °C until for RNA isolation.

RNA isolation was performed using TRIzol™ LS reagent kit according to the manufacture's instruction (Thermo Scientific, Mississauga, CA, U.S.A.). RQ1 RNase-Free DNase (Promega Corporation, Madison, WI, U.S.A.) was used to remove contaminant genomic DNA. DNase-treated RNA samples were separated into two parts: one for the negative control in quantitative PCR and one for RNA reverse transcription. cDNA libraries were generated using QuantiTect reverse transcription kit (Qiagen) and stored at – 20 °C until for use. Samples for RNA isolation were performed with three biological replicates.

### 4.2.6 Relative quantification of gene expression by quantitative PCR (qPCR)

qPCR amplification of *gcl* and *pho* (Teixeira et al., 2014) encoding for phospho-ketolase in *Lm. reuteri* was performed with primers that are listed in Table 4.1.

**Table 4.1** Primers used in RT-PCR.

| Targeted genes | Primer (forward, F; reverse, R) | Sequence (5'-3') |
|---|---|---|
| *pho* | *pho*, F | GTA ACC TTC AAG GAA TCC |
| | *pho*, R | CGT CTT TAC GCA TTC CTT G |
| *gcl1* | *gcl1*, F | CCT AGC AGT TCT GTA GGA GTT CG |
| | *gcl1*, R | GCC TGA TAT CTA CAA ATT GCT TGG G |
| *gcl2* | *gcl2*, F | CGA GTC CGG TTT CTG AAG ATATGC |
| | *gcl2*, R | GTG CCA TTA TCC ATG AAG TGC TTT AG |
| *gcl3* | *gcl3*, F | ACA AGG GCA AAT CAG CTC ATA CC |
| | *gcl3*, R | GAA GCC CGC TTC TTA GGA CG |

Specificity of all primers was analyzed by standard PCR with chromosomal DNA served as template. qPCR was carried out using a QuantiFast SYBR green master mixture (Qiagen) with a 7500 Fast Real Time-PCR System (Applied Biosystems, U.S.A.). The qPCR reaction mixture contained 12.5 µl of QuantiFast SYBR green master mix, 5 µl of 10 µM one pair of primers (forward and reverse), 1µl of cDNA template (~50 ng/µl), and 6.5 µl of RNase-free water. The qPCR amplification program was performed with a pre-denaturation stage at 95°C for 5 min; 40 cycles of two steps of denaturation at 95°C for 10 s, and anneal/extend at 60°C for 30 s, and elongation at 72°C for 30 s; followed by a melting stage with default settings. DNase-treated RNA samples of all cDNA samples and RNase-free water were used as negative controls. The *pho* gene was served as reference gene in the calculation of relative gene expression ratios; exponential-phase cultures of *Lm. reuteri* in mMRS were considered as reference conditions. The efficiencies of PCR reactions targeting pho and *gcls* were determined as described previously (Teixeira et al., 2014) and were 1.90, and 2.00, respectively. A mathematical model for the calculation of relative expression in RT-qPCR is as follows:

$$\Delta\Delta C_T = \text{Log}\frac{e_{gcl}\Delta C_T(\text{sourdough} - \text{mMRS})}{e_{pho}\Delta C_T(\text{sourdough} - \text{mMRS})}$$

Where e is PCR efficiency and $C_T$ value is threshold cycles for the amplification curves. Each cDNA sample for qPCR amplification was assayed in duplicate. Results are shown as means ± standard deviation of three biological replicates.

### 4.2.7 Synthesis of γ-glutamyl dipeptides by Gcls from *Lm. reuteri*

To determine the substrate specificity of Gcls in presence of a mixture of amino acids, the three enzymes were purified as described in Chapter 3. A mixture of 12 amino acids: Ile, Leu, Phe, Cys, Ala, Gly, Ser, Met, Pro, Gln, Glu, and Asp was prepared in 50 mM Trizma buffer with pH 9.0. This mixture (150 µl) was pipetted into a 225 µl of pre-warmed reaction cocktail containing 50 mM Trizma base, 40 mM ATP, 60 mM Glu, 40 mM $MgCl_2$ and 2 mM EDTA, pH 9.0, followed by addition of 75 µl of Gcl enzyme solution containing 94.5 µg of Gcl1, 66.0 µg of Gcl2 or 162.8 µg of Gcl3 to initiate the enzymatic reaction. The initial concentration of each amino acid in reaction was 9 mM. All reactions were performed at 43 °C for 2 h and terminated by an addition of 150 µl of 500 mM SSA. Precipitated proteins were removed by centrifugation at 13000 x g for 10 min; supernatant was transferred into a clean tube and store at – 20 °C until for quantitation of γ-glutamyl dipeptides. Reaction controls were prepared by addition of SSA prior to addition of the enzymes. Results are shown as means ± standard deviation of three biological replicates.

### 4.2.8 Quantitation of γ-Glutamyl Dipeptides by LC-MS/MS

γ-Glutamyl dipeptides using LC-MS/MS were similar quantitated as described by service of the Mass Spectrometry Facility of the Dept. of Chemistry of the University of Alberta (Lin et al., 2023). LC-MS/MS parameters for quantitation of 12 kokumi peptides are shown in Table 4.2.

**Table 4.2.** LC−MS/MS parameters for quantitation of γ-glutamyl dipeptides.

| Analyte | Ion transition (m/z) | Retention time (min) | Cone voltage (V) | Collision energy (V) |
|---|---|---|---|---|
| InfinityLab Poroshell 120 HILIC-Z, P column | | | | |
| γ-Glu-Cys | [a)]308.2/179.1 | 6.65 | 15 | 19 |
| γ- Glu-Gly | 205.1/76.05 | 6.57 | 15 | 13 |
| γ- Glu-Pro | 245.2/116.12 | 5.98 | 15 | 15 |
| γ- Glu-Ala | 219.2/90.08 | 6.10 | 15 | 14 |
| γ- Glu-Ser | 235.2/106.08 | 6.78 | 14 | 15 |
| γ-Glu-Gln | 276.2/147.1 | 6.88 | 14 | 16 |
| γ- Glu-Asp | 263.2/134.1 | 7.03 | 15 | 15 |
| γ-Glu-Glu | 277.2/148.1 | 6.90 | 15 | 16 |
| γ-Glu-Met | 279.2/150.2 | 5.15 | 15 | 16 |
| Luna Omega polar C18 column | | | | |
| γ-Glu-Phe | 295.3/166.2 | 4.17 | 15 | 18 |
| γ-Glu-Leu | 261.2/132.1 | 3.51 | 15 | 15 |
| γ-Glu-Ile | 261.2/132.1 | 3.28 | 15 | 15 |

[a)] γ-Glu-Cys alkylated with IAM was used in LC-MS/MS analysis.

Samples were adjusted to a pH of about 8.5 prior to the derivatization of γ-Glu-Cys. Twenty µl of 100 mM DTT (freshly made) was mixed with 200 µl of samples (pH >8.5). Subsequently, the mixture was incubated for 30 min at 50 °C to reduce the disulfide linkage, followed by an addition of 20 µl of 200 mM IAM to alkylate γ-Glu-Cys in dark at ambient temperature for 20 min; the resulting mixture was finally acidified to pH of 3.0 – 3.5 by 20 µl of 500 mM SSA. Two hundred µl of alkylated samples were concentrated to 10 µl using Savant SpeedVac vacuum concentrators (Thermo Scientific), then resuspended in the solution of 100 µl of 45% acetonitrile with 0.1% formic acid (FA, v/v) for HPLC-MS/MS analysis. γ-Glu-Cys standard was derivatized as described above. Standard mixtures of 9 γ-glutamyl dipeptides (γ-Glu-Ala, γ-Glu-Gly, γ-Glu-Ser, γ-Glu-Met, alkylated γ-Glu-Cys, γ-Glu-Pro, γ-Glu-Gln, γ-Glu-Glu, and γ-Glu-Asp) with

concentrations ranging from 0.05 to 100 ng/µl were prepared using the solution of 45% acetonitrile with 0.1% FA. γ-Glutamyl dipeptides were separated using an InfinityLab Poroshell 120 HILIC-Z, P column (2.7 µm, 50 × 2.1 mm, Phenomenex, Torrance, CA, U.S.A.) in a Wasters Acquity UPLC unit and detected by a Xevo TQ MS system (Waters Corporation, Milford, Massachusetts, U.S.A) with multiple reaction monitoring (MRM). Mobile phase A and B consisted of Milli-Q water with 0.1 % FA and 0.1 % ammonium formate ($NH_4FA$) and 90 % acetonitrile with 0.1 % FA and 0.1 % $NH_4FA$, respectively. Samples were eluted with flow rates and gradients as described (Lin et al., 2023). Data acquisition was interfaced to the Masslynx v4.1 software (Waters Corporation, Milford, Massachusetts, U.S.A).

To quantify γ-Glu-Ile, γ-Glu-Leu, and γ-Glu-Phe, 500 µl of samples were concentrated by 10 µl using SpeedVac vacuum concentrators, then resuspended by using 190 µl of Milli-Q $H_2O$ with 0.1% FA. The standard mixtures of γ-Glu-Ile, γ-Glu-Leu, and γ-Glu-Phe with different concentrations ranging from 0.001 to 1 ng/µl were prepared using Milli-Q $H_2O$ with 0.1% FA. γ-Glu-Ile, γ-Glu-Leu, and γ-Glu-Phe were separated using a Wasters Acquity UPLC unit equipped with a Luna Omega polar C18 column (1.6 µm, 50 × 2.1 mm, Phenomenex, Torrance, CA, U.S.A.) and detected by a Xevo TQ MS system (Waters Corporation, Milford, Massachusetts, U.S.A) with multiple reaction monitoring (MRM). Mobile phase A and B consisted of 0.1 % FA in Milli-Q water and 0.1 % FA in acetonitrile, respectively. Samples were eluted using the same flow rates and gradients as described (Lin et al., 2023). Data acquisition was interfaced to the Masslynx v4.1 software (Waters Corporation, Milford, Massachusetts, U.S.A).

### 4.2.9 Development of a bread model for sensory analyses

A bread model was developed as a sensory matrix, which consists of the following ingredients per 450 g of dry mass: 300 g of wheat starch, 150 g of skim milk powder, 210 g of

filtered tap $H_2O$, and 9 g of baking soda ($NaHCO_3$). To identify whether GSH is tasteless itself, bread models were made with or without an addition of 250 or 500 μmol/kg GSH. To analyze kokumi activity of GSH, GSH was added at 250 or 500 μmol/kg to bread models that additionally contained the basic tastants as follows: 20 mM/kg lactic acid, 30 mM/kg dextrose, 30 mM/kg fructose, and 2 mM/kg monosodium glutamate. To avoid a strong reaction of baking soda with lactic acid, the baking soda was mixed well with wheat starch and skim milk powder, following the addition of diluted lactic acid solution. The dough of the bread models was prepared using a spiral kneader at a slow speed for 1 min, then at a fast speed for 5 min. Each dough was evenly split into two parts and shaped by hand. The bread model was baked in a multideck oven (Bakers Pride, Lachine, QC, Canada) with forced air at a temperature of 160 °C for 40 min. The bread was cut into pieces after cooling down at room temperature. The bread crust was cut off; bread crumb was cut into 2 $cm^3$ pieces. Two pieces of each bread were placed in a covered plastic cup labeled with three-digit random numbers. The samples were stored at – 20 °C and thawed overnight at room temperature prior to the sensory analyses.

### 4.2.10 Sensory evaluation of bread models by a consumer panel

The sensory studies were reviewed and approved by the Research Ethics Board at the University of Alberta (study ID: Pro00114679). Sensory evaluation was performed in the sensory testing room at the Department of Agricultural, Food and Nutritional Science, University of Alberta. Triangle tests (Poste et al., 1991) were used to determine whether panellists correctly identifies the taste difference between samples with and without an addition of GSH. Two trials that used 250 and 500 μmol/kg GSH, respectively, were carried out in this study. Each trial had two sets of triangle tests: the first set contained three samples without the addition of basic tastants; the second set contained three other samples with the addition of basic tastants, respectively, to

evaluate the impact of GSH on the taste of bread models. Forty-four and forty-six of panelists were recruited randomly and irrespective of their bread consumption habits at the Agricultural & Forestry Centre, University of Alberta in two sensory panels, respectively. The panelists recruited were either students or researchers in University of Alberta and they were 24 – 35 years old. Six randomly assigned samples per session to each panelist and session. The red light in all sensory booths was used to mask the color differences. Water was provided to cleanse the palate between samples. The number of correct responses and the number of total responses were counted. Significance ($P < 0.05$) was determined as described in Poste et al. (1991).

### 4.2.11 Statistical Analysis

Data analysis for γ-glutamyl dipeptides in buffers with amino acids, dough, and bread was performed by one-way analysis of variance (ANOVA) and Independent-Samples T Test using IBM SPSS statistics 23. *P* value of ≤0.05 with Tukey adjustment for multiple comparisons was considered statistically significant. Data for sensory evaluation of bread models were analyzed using Statistical Chart 2 created by Poste et al (Poste et al., 1991). The number of the right judgment in the triangle test was compared to Chart which is the one-tailed with p=⅓ value. The statistical significance was $P< 0.05$.

## 4.3 Results

### 4.3.1 Synthesis of γ-glutamyl dipeptides by Gcls.

Gcl1, Gcl2 and Gcl3 differ with respect to the specificity to different acceptor amino acids (Chapter 3). To assess the formation of γ-glutamyl dipeptides in reactions where multiple amino acids are present, dipeptide formation was determined using a mixture of 12 amino acids that were selected based on substrate specificity of three Gcls with individual amino acids (Chapter 3). The results of enzymatic activity of three Gcls to 12 amino acids are shown in Figure 4.1.

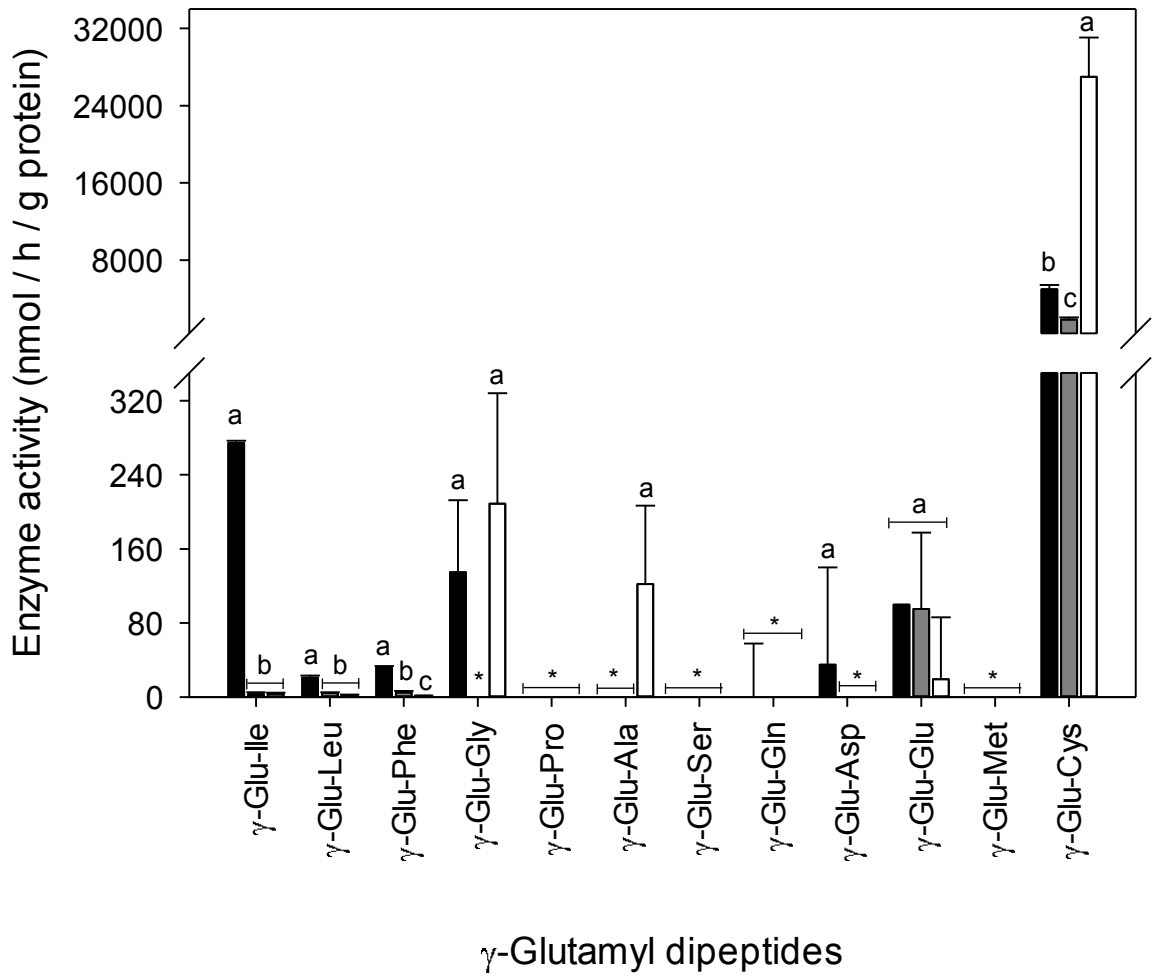



**Figure 4.1.** Quantitation of 12 γ-glutamyl dipeptides synthesized by γ-glutamyl cysteine ligases (Gcls) from *Lm. reuteri*. Colours indicate the activity of Gcl1 (black), Gcl2 (gray), and Gcl3 (white) to synthesize γ-glutamyl dipeptides. The detection limits of γ-Glu-Ala, γ-Glu-Gly, γ-Glu-Ser, γ-Glu-Met, alkylated γ-Glu-Cys, γ-Glu-Pro, γ-Glu-Gln, γ-Glu-Glu, and γ-Glu-Asp separated by an InfinityLab Poroshell 120 HILIC-Z, P column and of γ-Glu-Ile, γ-Glu-Leu, γ-Glu-Phe separated by a Luna Omega polar C18 column were 0.05 ng/µl and 0.1 ng/ml, respectively. "not detected" means the signal was lower than detection limits and was indicated with an asterisk.

The concentration of γ-Glu-Cys was highest after the reactions catalyzed by Gcl1, Gcl2, or Gcl3 compared to other products (Figure 4.1). All three Gcls thus exhibited high affinity to Cys. The activity of Gcl3 in forming γ-Glu-Cys was three times higher when compared to Gcl1 and Gcl2. All three Gcls additionally synthesized γ-Glu-Ile, γ-Glu-Leu, γ-Glu-Phe, and γ Glu-Glu but

not γ-Glu-Pro, γ-Glu-Ser, γ-Glu-Gln, and γ-Glu-Met. Gcl1 had a higher activity than Gcl2 and Gcl3 with the acceptor amino acids Ile, Leu, Phe, and Asp. Gcl1 was most active to Ile among three hydrophobic amino acids Ile, Leu, and Phe. γ-Glu-Gly was generated by Gcl1 and Gcl3 but not by Gcl2. The substrate specificity of the three Gcls from *Lm. reuteri* with mixtures of 12 amino acids thus differed from the specificity determined in reactions containing only one amino acid (Chapter 3).

### 4.3.2 Expression of Gcls during growth of *Lm. reuteri* in sourdough.

To determine the expression of Gcls during growth of *Lm. reuteri* LTH2584, LTH5448, and LTH5448Δ*gcl1* in sourdough, mRNA was quantified by RT-qPCR (Figure 4.2). *Lm. reuteri* LTH2584 repressed the expression of all three *gcl* genes during growth in sourdough. The expression of *gcl1* was downregulated during growth of *Lm. reuteri* LTH5448 in sourdough, but the expression level of *gcl2* was not altered after 48 h of fermentation. Similar to *Lm. reuteri* LTH2584, expression of *gcl2* by *Lm. reuteri* LTH5448Δ*gcl1* was downregulated during growth in sourdough. In summary, *gcl1*, *gcl2*, and *gcl3* from *Lm. reuteri* LTH2584, LTH5448, and LTH5448Δ*gcl1* were not overexpressed during sourdough fermentation (Figure 4.2).

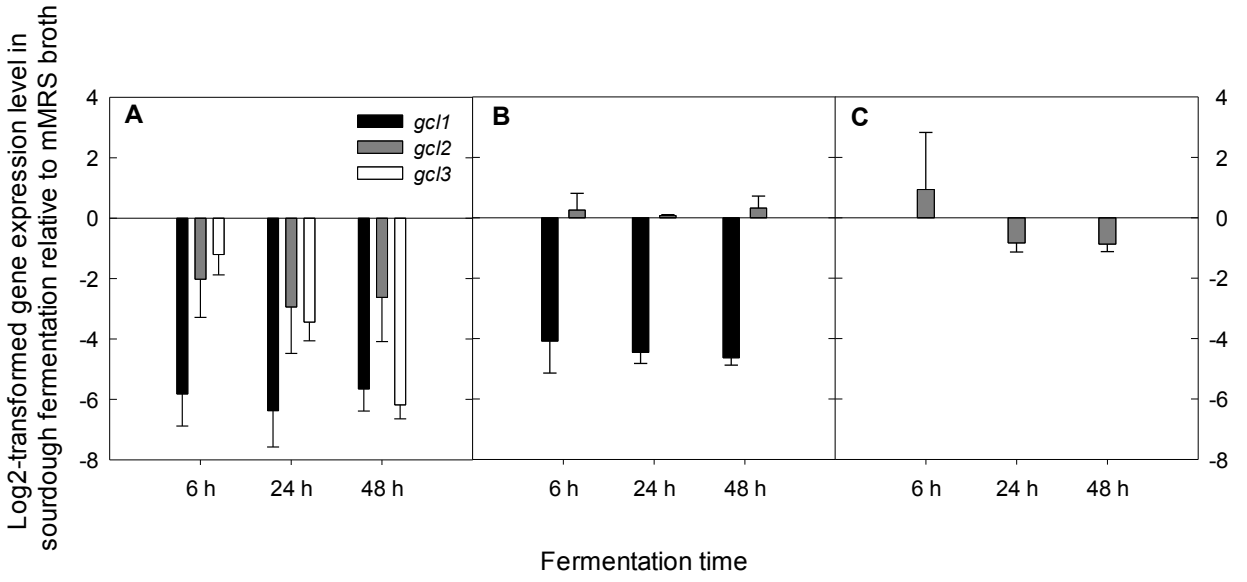



**Figure 4.2.** Effect of growth conditions on the relative expression of *gcl* genes in *Lm. reuteri* LTH2584 (A), LTH5448 (B), and LTH5448*Δgcl*1 (C), respectively. Colours indicate different genes of *gcl1* (black), *gcl2* (gray), and *gcl3* (white). Gene expression was quantified by RT-qPCR using *pho* as house-keeping gene and exponentially growing cells in mMRS as reference conditions.

### 4.3.3 Changes in pH and cell counts of sourdoughs

Changes in pH and cell count of sourdoughs were monitored during fermentation (Table 4.3). The pH of sourdoughs fermented with *Lm. reuteri* decreased from 5.76 – 5.78 to 3.61 – 3.71 after 24 h and increase slightly during further fermentation (Table 4.3). All four *Lm. reuteri* strains grew to high cell counts within 24 h and declined after 72 h. In chemically acidified sourdough, the pH remained unchanged during fermentation and viable bacterial counts remained below 2 log (cfu/g).

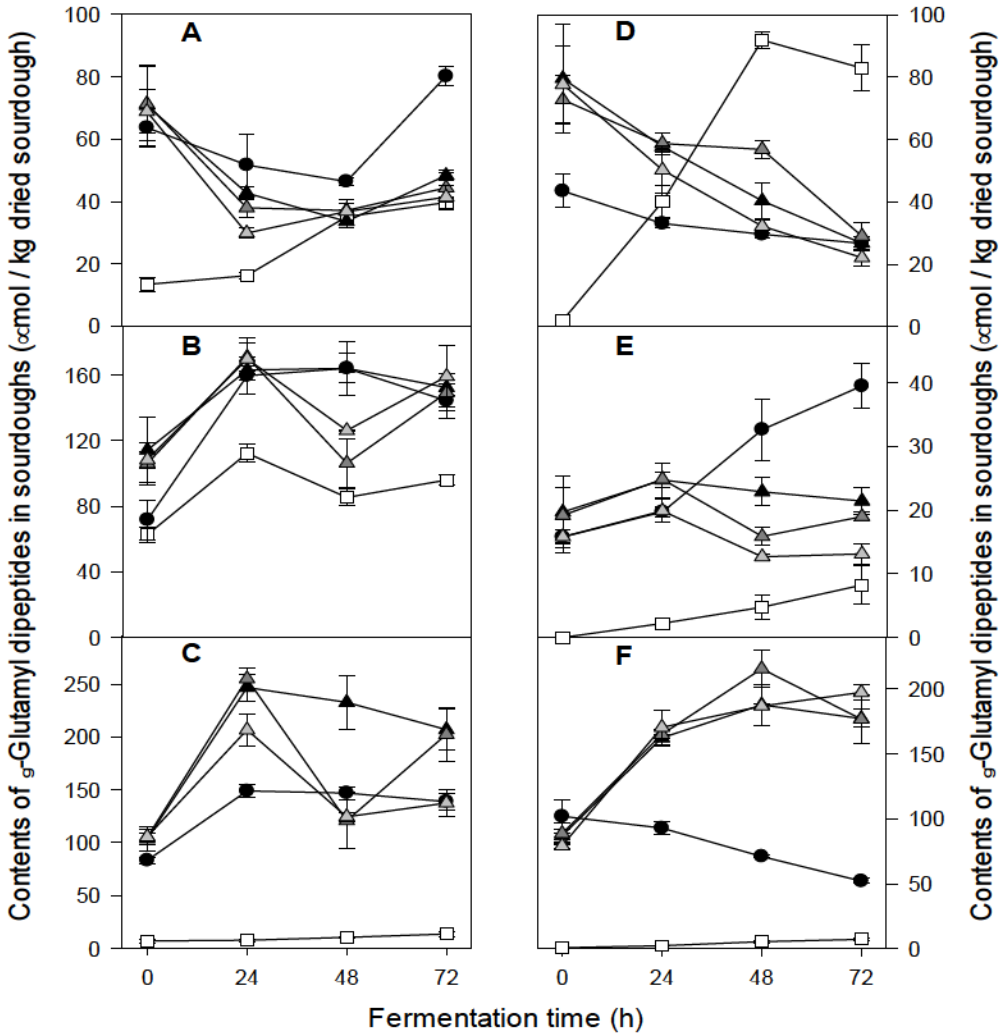
**Table 4.3.** Cell counts and pH of sourdoughs fermented with *Lm. reuteri* strains.

| Fermentation time | Chemically acidified | LTH2584 | LTH5448 | LTH544Δgcl1 | LTH544Δ*gcl1* Δ*gcl2* |
|---|---|---|---|---|---|
| | | | pH | | |
| 0 h | 3.51 ± 0.01 | 5.77 ± 0.01 | 5.78 ± 0.01 | 5.76 ± 0.01 | 5.78 ± 0.02 |
| 24 h | 3.52 ± 0.05 | 3.61 ± 0.04 | 3.71 ± 0.02 | 3.69 ± 0.01 | 3.69 ± 0.01 |
| 48 h | 3.45 ± 0.01 | 3.75 ± 0.02 | 3.78 ± 0.01 | 3.79 ± 0.01 | 3.82 ± 0.01 |
| 72 h | 3.50 ± 0.02 | 3.80 ± 0.01 | 3.84 ± 0.01 | 3.83 ± 0.02 | 3.88 ± 0.02 |
| 96 h | 3.55 ± 0.05 | 3.80 ± 0.02 | 3.88 ± 0.02 | 3.90 ± 0.02 | 3.92 ± 0.01 |
| | | | Cell Counts [Log (cfu/g)] | | |
| 0 h | <2 | 7.05 ± 0.06 | 8.07 ± 0.05 | 8.06 ± 0.11 | 8.21 ± 0.04 |
| 24 h | <2 | 9.63 ± 0.03 | 8.73 ± 0.09 | 8.43 ± 0.29 | 8.58 ± 53 |
| 48 h | <2 | 9.29 ± 0.26 | 8.46 ± 0.17 | 8.42 ± 0.29 | 8.34 ± 0.29 |
| 72 h | <2 | 7.72 ± 0.04 | 7.94 ± 0.18 | 7.77 ± 0.21 | 7.88 ± 0.19 |
| 96 h | <2 | 5.27 ± 0.09 | 5.42 ± 0.01 | 5.63 ± 0.05 | 5.64 ± 0.06 |

### 4.3.4 Quantification of γ-glutamyl dipeptides in sourdoughs

Twelve γ-glutamyl dipeptides were quantified in sourdough samples (Figure 4.3). The dipeptides were selected on the basis of the substrate specificity of the three Gcls (Chapter 3) and the description of kokumi activity of γ-glutamyl dipeptides (Zhao et al., 2016). In the initial stage of fermentation, the concentrations of γ-glutamyl dipeptides except γ-Glu-Ile in sourdoughs were higher than those in chemically acidified dough (Figure 4.3). This may represent carry over from the cultures in mMRS media. For some (γ-Glu-Cys, γ-Glu-Gly, γ-Glu-Ser, γ-Glu-Pro, γ-Glu-Met γ-Glu-Ile, γ-Glu-Leu) but not for all (e.g. γ-Glu-Ala, γ-Glu-Asp, γ-Glu-Glu) dipeptides, the increase during fermentation was greater in sourdoughs than in the chemically acidified control, indicating a contribution of microbial metabolism. The concentrations of γ-Glu-Ser, γ-Glu-Pro, γ-Glu-Met, γ-Glu-Ile, γ-Glu-Leu, and γ-Glu-Phe differed depending on whether *Lm. reuteri* LTH2584 or *Lm. reuteri* LTH5448 was used as inoculum, indicating strain-specific differences.

Substantial and consistent differences between *Lm. reuteri* LTH5448 and its mutants were observed for γ-Glu-Ile and γ-Glu-Leu, demonstrating that Gcl1 contributes to their formation in sourdough. *Lm. reuteri* LTH5448Δ*gcl1*Δ*gcl2* continued to accumulate γ-Glu-Cys and γ-Glu-Pro, indicating that enzymes other than γ-glutamyl cysteine ligases generate γ-glutamyl dipeptides. Taken together, these results document that cereal enzymes, γ-glutamyl-cysteine ligases and other microbial enzymes play a role in synthesis and degradation of γ-glutamyl dipeptides during sourdough fermentation.



(continued)

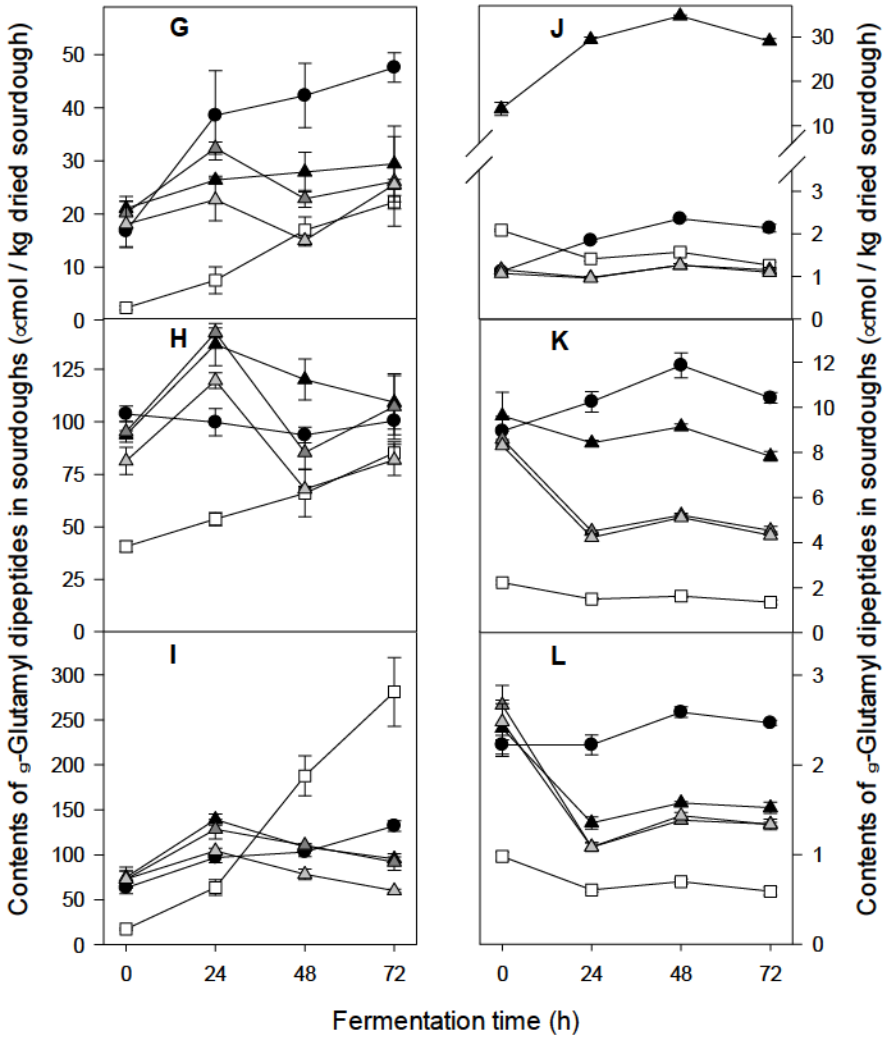



**Figure 4.3.** Contents of γ-glutamyl dipeptides in chemically acidified doughs (square, white) and sourdoughs fermented with *Lm. reuteri* LTH2584 (circle, black), LTH5448 (triangle, black), LTH5448 *Δgcl*1 (triangle, dark grey) or LTH5448Δ*gcl1*Δ*gcl2* (triangle, grey). A) γ-Glu-Gln; B) γ-Glu-Cys; C) γ-Glu-Gly; D) γ-Glu-Ala; E) γ-Glu-Ser; F) γ-Glu-Pro; G) γ-Glu-Met; H) γ-Glu-Asp; I) γ-Glu-Glu; J) γ-Glu-Ile; K) γ-Glu-Leu; and L) γ-Glu-Phe.

### 4.3.5 Quantification of γ-glutamyl dipeptides in bread dough and bread

To evaluate impacts of sourdoughs fermented with *Lm. reuteri* strains, proofing procedure, and baking process on the concentration of γ-glutamyl dipeptides, 12 γ-glutamyl dipeptides were

quantified in the doughs with or without an addition of dry sourdoughs fermented by *Lm. reuteri* LTH2584 and *Lm. reuteri* LTH5448Δ*gcl1*Δ*gcl2* and the corresponding breads (Table 4.4).

**Table 4.4.** Concentrations of γ-glutamyl dipeptides (μmol/kg) in bread doughs and breads.

| γ-Glutamyl dipeptides | LTH2584 bread | | LTH5448Δ*gcl1*Δ*gcl2* bread | | straight-dough bread | |
|---|---|---|---|---|---|---|
| | Bread dough | bread | Bread dough | bread | Bread dough | bread |
| γ-Glu-Cys | 68.91 ± 6.49$^{A}$ | 83.57 ± 13.34$^{b}$ | 79.13 ± 5.52$^{A, *}$ | 112.72 ± 3.32$^{a}$ | 44.30 ± 6.63$^{B, *}$ | 95.44 ± 14.07$^{ab}$ |
| γ-Glu-Gly | 27.87 ± 3.42$^{A}$ | 27.43 ± 5.49$^{b}$ | 29.76 ± 1.81$^{A, *}$ | 38.71 ±3.58$^{ab}$ | 20.60 ± 1.37$^{B, *}$ | 47.47 ± 10.10$^{a}$ |
| γ-Glu-Pro | 83.12 ± 8.44$^{A, *}$ | 26.81 ± 2.65$^{b}$ | 96.86 ± 4.33$^{A, *}$ | 44.62 ± 2.26$^{a}$ | 13.37 ± 2.39$^{B, *}$ | 5.24 ± 0.12$^{c}$ |
| γ-Glu-Ala | 78.40 ± 10.47$^{*}$ | 36.79 ± 13.37$^{b}$ | 62.98 ± 0.42$^{*}$ | 50.27 ± 1.45$^{ab}$ | 52.67 ± 15.19 | 66.94 ± 4.97$^{a}$ |
| γ-Glu-Ser | 5.84 ± 1.09$^{A, *}$ | 2.18 ± 0.73 | 2.81 ± 0.32$^{B, *}$ | 1.51 ± 0.63 | 1.40 ± 0.84$^{B}$ | 1.34 ± 0.47 |
| γ-Glu-Gln | 20.90 ± 2.15$^{A}$ | 15.37 ± 6.02 | 13.14 ± 4.34$^{B}$ | 7.18 ± 1.18 | 6.81 ± 1.10$^{B}$ | 8.95 ± 2.85 |
| γ-Glu-Asp | 35.76 ± 3.93 | 38.46 ± 4.24$^{b}$ | 33.48 ± 2.31 | 28.85 ± 4.31$^{b}$ | 40.29 ± 6.29$^{*}$ | 78.03 ± 16.67$^{a}$ |
| γ-Glu-Glu | 19.59 ± 0.76 | 23.89 ± 8.89 | 17.60 ± 2.12$^{*}$ | 11.74 ± 1.42 | 15.58 ± 2.02 | 22.52 ± 7.71 |
| γ-Glu-Met | 25.73 ± 1.88$^{A, *}$ | 2.22 ± 0.59 | 13.06 ± 0.87$^{B, *}$ | 1.49 ± 0.37 | 3.84 ± 1.87$^{C}$ | 1.11 ± 0.61 |
| γ-Glu-Ile | 2.42 ± 0.10 | 2.53 ± 0.02$^{ab}$ | 2.54 ± 0.12 | 2.41 ± 0.06$^{b}$ | 2,49 ± 0.28 | 2.59 ± 0.05$^{a}$ |
| γ-Glu-Leu | 6.84 ± 0.24 | 6.70 ± 0.21$^{a}$ | 8.02 ± 0.54 | 6.89 ± 0.17$^{a}$ | 6.78 ± 0.70 | 6.18 ± 0.09$^{b}$ |
| γ-Glu-Phe | 2.66 ± 0.12$^{B}$ | 2.02 ± 0.03$^{b}$ | 3.19 ± 0.24$^{A}$ | 2.17 ± 0.03$^{a}$ | 2.18 ± 0.24$^{B}$ | 1.87 ± 0.02$^{c}$ |

Significant differences ($P$<0.05) among bread doughs or breads are indicated with an uppercase superscript or a lowercase superscript, respectively. Significant differences ($P$<0.05) between bread dough and bread are indicated with an asterisk.

In all three doughs, γ-Glu-Cys, γ-Glu-Gly, γ-Glu-Pro, γ-Glu-Ala, and γ-Glu-Asp were the most abundant γ-glutamyl dipeptides. The concentration of 7 of the 12 γ-glutamyl dipeptides differed between straight dough and bread dough with sourdough and the concentration was higher in both or one of those doughs containing sourdough (Table 4.4). For the three dipeptides where the concentrations of γ-glutamyl dipeptides differed between sourdoughs fermented with *Lm. reuteri* LTH2584 and *Lm. reuteri* LTH5448, the concentration was higher in those doughs containing sourdough fermented with *Lm. reuteri* LTH2584. Baking changed the concentrations of 8 of the 12 γ-glutamyl dipeptides in one or more of the breads when compared to the corresponding doughs sampled just prior to baking (Table 4.4). The concentration of only one

peptide, γ-Glu-Pro, was lower in all three breads when compared to the corresponding doughs. The concentration of γ-Glu-Ala, γ-Glu-Ser and γ-Glu-Met decreased in the bread with sourdough but not the straight dough bread. The concentration of γ-Glu-Asp increased only in straight dough bread. γ-Glu-Leu and γ-Glu-Phe were the only dipeptides with a higher concentration in sourdough bread when compared to straight dough bread. γ-Glu-Cys, γ-Glu-Pro and γ-Glu-Phe were lower in breads produced with *Lm. reuteri* TLH2584 sourdough when compared to breads produced with *Lm. reuteri* LTH5448Δ*gcl1*Δ*gcl2* sourdough.

### 4.3.6 Sensory analysis of a bread model supplemented with the kokumi-active glutathione

The impact of γ-glutamyl peptides on the sensory properties of bread was determined with glutathione, the only γ-glutamyl peptide which is available commercially as food grade ingredient. To avoid interference of glutathione that is present in wheat flour as well as baker's yeast, the bread model was composed of skim milk powder and wheat starch and was leavened chemically. Glucose, fructose, monosodium glutamate (MSG) and lactate were added, or not, to concentrations matching those in sourdough bread (Zhao et al., 2015; Müller et al., 2021). The results of sensory evaluation of GSH impact on the taste of bread models are shown in Table 4.5. In the first trial that used 250 μmol/kg GSH, the proportions of correct responses in total responses to the taste of bread models without and with tastants were 29.5% and 38.6%, respectively. The corresponding *p*-values (Poste et al., 1991) were 0.753 and 0.275, respectively, indicating that irrespective of the presence of tastants, glutathione did not impact the taste of bread. In the second trial, the concentration of GSH was increased to 500 μmol/kg but also this glutathione concentration did not impact the taste of bread (Table 4.5).

**Table 4.5.** Sensory evaluation of bread models using triangle tests.

| Triangle tests | Proportion of correct responses in total responses (%) | Observed p-value | Significant at $p<0.05$ (YES/NO) |
|---|---|---|---|
| bread model with or without 250 μmol/kg GSH | 29.5 | 0.753 | NO |
| bread model with tastants and with or without 250 μmol/kg GSH | 38.6 | 0.275 | NO |
| bread model with or without 500 μmol/kg GSH | 21.7 | 0.970 | NO |
| bread model with tastants and with or without 500 μmol/kg GSH | 30.4 | 0.713 | NO |

Tastants: 20 mM/kg lactic acid, 30 mM/kg dextrose, 30 mM/kg fructose, and 2 mM/kg monosodium glutamate; saltiness was contributed by the sodium of baking soda.

## 4.4 Discussion

γ-Glutamyl peptides are potent kokumi substances. The kokumi threshold of γ-glutamyl peptides varies depending on the food matrices, which contain different concentrations of taste compounds, and ranges from less than 5 μmol/kg to 2.5 mmol/kg (Ueda et al., 1997; Zhao et al., 2016). The present study determined that total concentration of kokumi γ-glutamyl dipeptides ranged from 250 – 350 μmol/kg in sourdough or straight-dough breads (Table 4.4), which is higher than the expected kokumi threshold in fermented foods (Zhao et al., 2016). GSH, a γ-glutamyl tripeptide that has been characterized as kokumi active in model chicken and beef solutions (Ohsu et al., 2010; Ueda et al., 1997), did not impact the taste of bread models at the concentration of 500 μmol/kg. However, this observation does not exclude a contribution of γ-glutamyl peptides on the taste of sourdough bread. The concentrations of γ-Glu-Gln, γ-Glu-Glu, γ-Glu-Gly, and γ-Glu-Leu in sourdough or straight-dough bread are above their kokumi threshold in cheeses (Toelstede et al.,

2009). The concentration of GSH in the bread model, 500 μmol/kg or 0.015% GSH, is likely below the kokumi threshold. The taste threshold of GSH in umami solutions with 0.05% glutamate was determined as 0.04% (w/v) (Ueda et al., 1997). In addition, the concentration of GSH in bread models might decrease after baking wherein GSH can be converted to pyroglutamic acid and cyclocysteinylglycine without kokumi activity (Ueda et al., 1997). However, the presence of kokumi active peptides in plant foods constrained the selection of the compounds that were used in the bread model. Yeast, gluten proteins, and pulse proteins all contain glutathione or γ-glutamyl dipeptides that would confound the results (Guha and Majumder, 2022; Tang et al., 2017). The kokumi thresholds of γ-Glu-Leu and γ-Glu-Val as well their threshold values for interaction with Calcium-sensing receptors were reported to be 100-times lower when compared to glutathione (Brennan et al., 2014; Ohsu et al., 2010). Glutathione is, however, the only kokumi-active peptide that is available in food grade quality and in sufficient quantity to prepare samples for two sensory panels with more than 40 panelists. The use of casein proteins to replace wheat gluten makes the bread model exhibit a creamy, milky and tangy flavour that are different from the flavour of bread, interfering the sensory evaluation of bread models. Thus, it remains a necessity to optimize the bread model for the sensory evaluation of γ-glutamyl dipeptide impact on the taste of bread.

Gcl activity of *Lm. reuteri* was downregulated (*gcl1*) or not over-expressed (*gcl2, gcl3*) during growth in sourdoughs. The substrate supply impacts the expression of genes related to the proteolytic system of lactic acid bacteria (Hebert et al., 2000; Guédon et al., 2001). The sourdough system that is rich in peptides including γ-glutamyl dipeptides (Zhao and Gänzle, 2016) may repress the expression of Gcls of *Lm. reuteri*. In particular, *gcl1* was repressed during growth in sourdough. Gcl1 exhibits stronger activity to hydrophobic amino acids when compared to Gcl2 and Gcl3 (Figure 4.1). Despite these low levels of expression when compared to mMRS media,

Gcl1 contributed to the formation of γ-Glu-Ile and γ-Glu-Leu in sourdough. γ-Glu-Leu is among the dipeptides with the lowest kokumi threshold (Zhao et al., 2016); the use of isogenic mutants in the present study firmly attributes its formation in sourdough to microbial γ-glutamyl-cysteine ligases.

γ-Glu-Cys was the main product of all three Gcl enzymes when multiple amino acids are present as equal amounts in enzymatic reactions. However, the accumulation of γ-Glu-Cys in sourdoughs and in sourdough bread was irrespective of the activity of bacterial Gcls. This indicates multiple other sources of enzymes from yeast or wheat that are capable of γ-glutamyl transpeptidase activity, or of proteolytic glutathione degradation to γ-Glu-Cys.(Thompson et al., 1964; Q. Li et al., 2022). The present study showed that the synthesis and degradation of γ-glutamyl dipeptides were attributed to the activity of cereal enzymes and microbial enzymes during sourdough fermentation. Plants contain γ-glutamyl transpeptidase that synthesize γ-glutamyl dipeptides (Thompson et al., 1964). The resulting γ-glutamyl transpeptidase from wheat may synthesize γ-glutamyl dipeptides from glutathione or free amino acids in sourdough fermentation. Proteolysis generating amino acids and peptides may thus play an important role in the accumulation of γ-glutamyl dipeptides during sourdough fermentation (Zhao and Gänzle, 2016). The addition of fungal protease combined with sourdough fermentation extends the degradation of gluten proteins, but the enzymes with γ-glutamyl transpeptidase activity present in commercial fungal protease preparation may additionally contribute to the synthesis of γ-glutamyl dipeptides (Tomita et al., 1989). The composition and concentration of amino acids and peptides released from gluten proteins are strongly influenced by the catalytic specificity of cereal enzymes, fungal protease, and peptidase from *Lm. reuteri* (Gänzle et al., 2008; da Silva, 2017). The resulting amino acids and peptides served as substrates of cereal or microbial enzymes, subsequently impacting

the accumulation of γ-glutamyl dipeptides in sourdoughs. The formation of γ-Glu-Ser, γ-Glu-Pro, γ-Glu-Met, and γ-Glu-Phe in sourdoughs showed strain-specific differences, but it remains unknown which enzymes are responsible for their accumulation by *Lm. reuteri*.

Thermal treatment strongly impacted the concentration of γ-glutamyl dipeptides such as γ-Glu-Cys/Gly/Pro/Ala/Ser/Asp/Glu/Met, similar to a previous study where baking increased the concentration of GSH in the bread (Won et al., 2021). Yeast cells contain γ-glutamyl dipeptides that may be released during baking, and biosynthetic pathways of γ-glutamyl dipeptides (Q. Li et al., 2022; Sofyanovich et al., 2019). In contrast, γ-Glu-Pro was degraded after baking. Baking processing also causes a degradation of tripeptides with two proline residues at the C-terminus (Zhao et al., 2013). The decrease of γ-glutamyl dipeptides may be related to Maillard reaction during baking or enzymatic degradation by γ-glutamyl peptidases (Zhao et al., 2013; Akhtar et al., 2010). Although the concentrations of several γ-glutamyl dipeptides including γ-Glu-Cys/Gly/Pro/Ser/Met/Phe in one or both bread doughs with sourdough were higher than straight dough, the addition of 10% sourdoughs did not increase the concentration of total γ-glutamyl dipeptides in bread.

## 4.5 Conclusion

All three Gcl enzymes in vitro exhibit the highest activity to Cys among 12 amino acids, but the formation of γ-Glu-Cys is irrespective of Gcls of *Lm. reuteri* in sourdough fermentation. The synthesis of γ-glutamyl dipeptides during sourdough fermentation was attributed to the activity of cereal enzymes, Gcls of *Lm reuteri* and other microbial enzymes. Gcl1 mediates the synthesis of γ-Glu-Ile and γ-Glu-Ile in sourdoughs. Baking processing strongly influences the concentration of γ-glutamyl dipeptides in bread. The use of 10% sourdough did not improve the total amount of 12

γ-glutamyl dipeptides in bread. The impact of kokumi γ-glutamyl dipeptides on the taste of bread remains to be investigated further.

# Chapter 5. The effect of germination and *Bacillus* spp. on the ripening of plant cheese analogues

## 5.1 Introduction

Consumer demand for plant-based foods including meat and dairy analogues has been increasing in recent years owing to concerns over human health, environmental sustainability, and animal welfare (McClements and Grossmann, 2022). This growing demand incentivises the development of new plant-based food products among which plant-based dairy analogues play an important role (Grossmann and McClements, 2021; Pua et al., 2022). Plant-based cheese analogues, which are subsequently referred to as plant cheese, are produced from mixtures of pure and functional plant ingredients such as plant proteins, plant oils/fats, and starches, etc., or generated directly from whole plant raw materials such as legumes, grains, and nuts. Plant cheeses are formulated either with or without the addition by fermentation cultures (Grossmann and McClements, 2021; Pua et al., 2022). Legumes are frequently used as raw materials for production of plant cheeses, owing to their high content of proteins and fat, low cost, and good functionalities (Mefleh et al., 2021).

Comparable to cheese production, acidification by lactic acid bacteria transforms pulse protein matrix to a cheese-like gel (Masia et al., 2022) and proteolytic activity during ripening releases taste-active amino acids and peptides (Yao et al., 2021). Phenolic compounds in plants have antinutritive activity or may impart astringency and/or bitter taste (Yan and Tong, 2023), but can also be converted by food fermenting lactobacilli to metabolites that are beneficial to human health or improve the flavor of foods (Gaur and Gänzle, 2023). Heterofermentative lactic acid bacteria also reduce the lipid oxidation aldehydes that cause the beany flavor of pulse proteins (Sugahara et al., 2022; Vermeulen et al., 2007). The ingredients of plant-based cheese analogues

also contain starch and non-starch polysaccharides but their hydrolysis during ripening has not been described.

Enzymes have been used as an effective tool to improve texture and flavor of plant cheese (Pua et al., 2022). Commercial hydrolytic enzymes improved the flavour and texture of soy cheeses (Li et al., 2013). Germination of plant seeds activates endogenous enzymes of seeds such as amylase and protease (Fernandez and Berry, 1988; Nkhata et al., 2018; G. Zhang et al., 2015). Short-time germination of soy beans improved the quality of resulting soymilk (Jiang et al., 2013). However, germination also favors the growth of foodborne pathogens from contaminated seeds or the environment (Dechet et al., 2014; Yang et al., 2013). The use of "rejuvelac" thus carries substantial risks for food safety (Schmitt et al., 2018).

Soy products such as tofu, *douchi* and *sufu* (fermented tofu) have been traditionally consumed in East Asia (Han et al., 2001b; L. Zheng et al., 2020). The fermentation of *sufu* includes a pre-fermentation stage where bacilli or mycelial molds produce hydrolytic enzymes to release sugars and amino acids that serve as substrates for microbial growth and flavour formation in the post-fermentation phase. Bacilli are plant endophytes and thus widely occur in plants and plant foods (Robinson et al., 2016). The endospores of bacilli viable in pasteurized products (Berendsen et al., 2016; Setlow, 2006). *Bacillus cereus* causes foodborne diseases in humans but other *Bacillus* spp. such as *Bacillus subtilis* var. natto are safe fermentation microbes (André et al., 2017; Li et al., 2023b). Strains of the *B. subtilis* group produce amylases, proteases, glutaminase, β-glucosidases, phytase, or α-galactosidase to decompose the raw materials or generate flavor compounds during food fermentation (Li et al., 2023b) but their use in plant cheeses has not been explored. It was therefore the aim of this study to investigate the microbiological and biochemical impact of germination and of *Bacillus* spp. on the ripening of plant-based cheese analogues.

Germinated or ungerminated soy beans or lupine seeds served as raw materials to produce cheese analogues that were ripened for 90 d. Seeds used were germinated with lactic acid bacteria (LAB) only or with LAB and *Bacillus* sp. as protective cultures.

## 5.2 Material and methods

### 5.2.1 Bacterial strains and growth conditions

Lactobacilli strains used in this study included *Lantiplantibacillus plantarum* TMW1.460 (NZ_WEZR01000000), *Lacticaseibacillus paracasei* FUA3413 (JANDJR000000000), *Levilactobacillus hammesii* LP38$^{T}$ (AZFS00000000), *Furfurilactobacillus milli* FUA3430 (JAIWJF000000000), *Limosilactobacillus reuteri* TMW1.656 (JOSW02000000), and *Lentilactobacillus buchneri* FUA3252 (JAUAPU000000000). They were grown in modified deMan-Rogosa-Sharpe (mMRS) medium under microaerophilic conditions at 30 °C; *Lactococcus lactis* FUA3228 (JAUAPV000000000) was grown in M17 medium (Sigma-Aldrich, St. Louis, MO, U.S.A.) under microaerophilic conditions at 30 °C. *Bacillus velezensis* FUA2155 (SDKI00000000) and *Bacillus amyloliquefaciens* Fad 82 (SDKG00000000) were incubated in Luria-Bertani (LB) medium (BD, Mississauga, Ontario, Canada) under aerobic conditions at 37 °C. To prepare inocula for fermentation, cells from overnight cultures were washed twice with equal volumes of 0.85% saline.

### 5.2.2 Materials and chemicals

Soy beans were purchased from a local supermarket; lupine seeds were kindly provided by Lumi Foods (Vancouver, Canada). Galactose, glucose, fructose, sucrose, melibiose, raffinose, stachyose, verbascose, ninhydrin, potassium iodate, 3, 5-dinitrosalicylic acid (DNS), phenol, potassium sodium tartrate tetrahydrate, and other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

### 5.2.3 Screening starter cultures to acidifying the soymilk

Soy beans were pre-treated for 30 min with 0.2% chlorine solution (1:3 w/v), washed two times with sterile water, and soaked with sterile $H_2O$ (1:5 w/v) for 2 h, and then blended by a KitchenAid blender for 3 min, followed by the filtration using sterile cheese cloth to obtain soymilk. One ml of inoculum was added into 40 ml of soymilk to obtain an initial cell concentration of ~$10^7$ cfu/ml, the inoculated soymilk was then incubated at 30 °C. Three ml of soymilk was collected after 0, 6, and 12 h fermentation for measurement of the pH.

### 5.2.4 Identification of bacteria in lupine seeds

To assay the effect of chlorine or LAB on plant microbiota during germination, lupine seeds were pre-treated with or without 0.2% chlorine solution, and soaked with water, or with water containing a cell suspension of about $10^7$ cfu/mL each of *Lp. plantarum* and *Lc. lactis*. Excess water drained and lupine seeds were germinated in a sterile container with 100% relative humidity (rH) at 30 °C for 2 d. Lupine milk was produced from germinated seeds with the same protocol that is described above for ungerminated seeds. Cell counts were determined with lupine seeds or fresh prepared after germination using agar plates prepared with LB, MRS (BD Difco, Sparks, MD, U.S.A.), M17, and Violet Red Bile Glucose (Sigma-Aldrich, St. Louis, MO, U.S.A.) media. Bacteria in all lupine milk were also identified by sequencing of full length 16S rRNA gene amplicons as outlined below. Milk samples were stored at – 20 °C until the use for DNA isolation. Three biological replicates were performed for each sample preparation.

### 5.2.5 Preparation and ripening of soy and lupine cheeses

Soy beans or lupine seeds were pre-treated with 0.2% chlorine solution for 30 min, followed by soaking with the cell suspension of *Lp. plantarum* and *Lc. lactis* with or without *B. velezensis* or *B. amyloliquefaciens* for 2 h. The cell count of each strain in soaking cultures ranged

from $10^7$ to $10^8$ cfu/ml. Soaked soy beans or lupine seeds were germinated under the condition as described above, then blended into the plant milk after addition of sterile $H_2O$ in a ratio of 1:5 (w/v). As a control of germination, the plant milk was also prepared from soy beans or lupine seeds that were pre-treated with 0.2% chlorine solution but were not germinated. The initial cell counts of *Lp. plantarum* and *Lc. lactis* in milk from ungerminated seeds are shown in Table 5.1.

**Table 5.1.** Cell counts of *Lp. plantarum*, *Lc. lactis*, and *Bacillus* spp. in plant milk from ungerminated or germinated seeds: with LAB only, or with LAB and *B. velezensis* or with *B. amyloliquefaciens*.

| | **Cell counts [log(cfu/ml)]** | | |
|---|---|---|---|
| **Samples** | ***Lp. plantarum*** | ***Lc. lactis*** | ***Bacillus* spp.** |
| Soymilk | | | |
| ungerminated | 8.0 ± 0.1 | 7.8± 0.4 | -- |
| germinated with LAB | 8.4 ± 0.3 | 7.8 ± 0.4 | -- |
| germinated with LAB and *B. velezensis* | 7.6 ± 0.1 | 6.8 ± 0.5 | 7.8 ± 0.1 |
| germinated with LAB and *B. amyloliquefaciens* | 8.5 ± 0.1 | 7.7 ± 0.2 | 7.7 ± 1.2 |
| Lupine milk | | | |
| ungerminated | 7.9 ± 0.0 | 7.0 ± 0.4 | -- |
| germinated with LAB | 7.6 ± 0.4 | 6.2 ± 0.5 | -- |
| germinated with LAB and *B. velezensis* | 6.8 ± 0.5 | 6.6 ± 0.2 | 7.1 ± 0.0 |

After 12 h of soy or lupine milk acidification at 30 °C, proteins were precipitated and harvested by centrifugation at 3500 x g for 10 min to obtain the soy or lupine cheeses. Subsequently, the cheese samples were ripened at 8 °C and rH 0.88 and collected after 0, 14, 45, and 90 d. Cell counts were assayed with fresh samples using LB, MRS, and M17 agar plates. The colony morphology of all colonies on the agar plates was compared to the colony morphology of the inocula. As described below, sequencing of 16S rRNA genes amplified from community DNA was used to verify conclusions based on the morphology of colonies on agar plates. The freezer-

dried samples were stored at – 20 °C prior to use for the analysis of carbohydrates, free amino nitrogen, SDS-page gel, and metagenomic sequencing of bacteria. The preparation and ripening of soy or lupine cheese were performed with three biological replicates.

### 5.2.6 Characterisation of mono-, di-, and oligosaccharides in milk and cheese samples

Soy or lupine milks were diluted 100-fold using Milli-Q $H_2O$ prior to analysis. To characterize the carbohydrates the in cheese samples, ~ 0.05 g of dry soy or lupine cheese was homogenized with 1ml of sodium phosphate buffer (100 mmol $L^{-1}$, pH 7.4) by the incubation at 25 °C with agitation 250 rpm for 1 h; the supernatant was obtained by centrifugation at 17,000 x g for 10 min, and diluted 10-fold prior to determination. Mono-, di-, and oligosaccharides were quantified by HPAEC-PAD using a Carbopac PA20 column coupled to an ED40 chemical detector on a Dionex ICS 6000 unit (Dionex, Oakville, Canada). Mobile phase A, B, and C consist of Milli-Q $H_2O$, 0.2 M sodium hydroxide, and 1 M sodium acetate, respectively. Samples were eluted at a flow of 0.25 ml $min^{-1}$ at the following gradients: 0 – 30 min, 15% B, 0 – 1.8% C; 30 – 80 min, 15% B, 1.8 – 20% C; 80 – 90 min, 15 – 0% B, 20 – 50% C; 90 – 95 min, 0 – 40% B, 50 – 0% C; 95 – 105 min, 40 – 80% B, 0% C; 105 –110 min, 80 – 15% B, 0% C; 110 – 130 min, 15% B, 0% C. Galactose, glucose, fructose, sucrose, melibiose, raffinose, stachyose, and verbascose solutions with the concentrations from 0.1 to 10.0 mg $L^{-1}$ served as external standards.

### 5.2.7 Determination of water-soluble polysaccharides in milk and cheese samples

Polysaccharides were extracted and hydrolyzed as described (Schwab et al., 2008). Milk or cheese samples were mixed or suspended with distilled $H_2O$, then incubated at 80 °C for 2 h. Solids were removed by centrifugation at 5000 x g for 10 min; supernatants were added into 2 volumes of 100% chilled ethanol, followed by an incubation at 4 °C for overnight to ensure the

polysaccharides precipitated completely. Polysaccharide precipitates were hydrolyzed in 1 ml of 1 M $H_2SO_4$ at 80 °C for 2 h.

Reducing sugars in polysaccharide hydrolysates were quantified using DNS method (Miller, 1959). The modified DNS solution was prepared as the following ingredients per 500 ml: 124.18 g of potassium sodium tartrate tetrahydrate, 131 ml of 2 M NaOH, 3.15 g of 3, 5-dinitrosalicylic acid, 2.5 g of phenol, and 2.5 g of sodium bisulfite. One hundred and sixty µl of hydrolysate was mixed with 40 µl of 10 M NaOH, then reacted with 150 µl of DNS solution at 100 °C for 5 min. After cooling to room temperature, the reaction mixture was adjusted to 2.5 ml using distilled $H_2O$. The absorbance was determined at 540 nm. Glucose solutions ranging from 0.1 to 1.0 mg $L^{-1}$ were used as external standards.

### 5.2.8 Quantification of free amino nitrogen (FAN) in ripened cheese samples

The content of free amino nitrogen in ripened cheese was determined using the ninhydrin method (Thiele et al., 2002). Free amino nitrogen molecules were extracted from ~ 0.05 g of dry cheese that was homogenized with 1ml of sodium phosphate buffer (100 mmol $L^{-1}$, pH 7.4). Two µl of the aqueous extract was mixed with 198 µl of distilled $H_2O$, followed by the addition of 100 µl of ninhydrin solution. The reaction mixture was incubated at 100 °C for 16 min, cooled to room temperature for 20 min, and finally mixed with 500 µl of $KIO_3$ solution. The absorbance was measured at 570 nm with a microplate reader (Varioskan Flash, Thermo Electron Corporation, CA, USA). Glycine served as an external standard.

### 5.2.9 DNA isolation and full length 16S rRNA gene sequencing

The identity of plant cheese ripening microbiota with inocula was verified by sequencing of full length 16S rRNA gene amplicons. DNA was isolated from ~ 0.1 g of dry cheese sample, which was homogenized with 1 ml of sterile saline $H_2O$ at 25 °C with agitation 250 rpm for 30

min and centrifuged at 10 x g for 2 min to remove solids. The supernatant was transferred into the clean tube, followed by centrifugation at 10000 x g for 5 min to obtain cell pellets. DNA was isolated from cell pellets using a DNeasy Blood and Tissue Kit (Qiagen, Mississauga, ON, Canada). Nanopore sequencing technology was used to amplify and analyze 16S rRNA genes. Amplicon libraries for sequencing were prepared using 16S Barcoding Kit 1-24 (SQK-16S024) according to the instructions of manufacturer (Oxford Nanopore Technologies, Oxford Science Park, Oxford, United Kingdom). DNA libraries were mixed with sequencing reagents provided by Flongle Sequencing Expansion Kit (EXP-FSE001), then loaded into a R9.4.1 Flongle flow cell according to the instructions of manufacturer (Oxford Nanopore Technologies). Data acquisition and high accuracy basecalling were carried out with MinKNOW v22.12.7 and Guppy v6.4.6, respectively; the basecalled data was analyzed in EPI2ME Labs wf-metagenomics v2.0.8. software using NCBI 16S and 18S rRNA database.

### 5.2.10 Genomic DNA isolation and whole genome sequencing

The genomes of *Lc. lactis* FUA3228 and *Lt. buchneri* FUA3252 were sequenced and annotated in this study. gDNA was isolated using Wizard Genomic DNA Purification Kit following the instructions provided by the manufacture (Promega, Madisson, Wisconsin, USA). Library DNA was prepared using the Nanopore Native Barcoding Kit (SQK-NBD114.24) and sequenced on the MinION platform using the R10.4.1 flow cell (Oxford Nanopore Technologies). Data was basecalled in super-high accuracy mode using Guppy v6.4.8. The genome was *de-novo* assembled by Flye, annotated by Prokka, and polished by Medaka using wf-bacterial-genomes v0.2.12 available in EPI2ME Labs (Oxford Nanopore Technologies).

### 5.2.11 Statistical analysis

Data analysis for the carbohydrate concentration of unripened plant cheese, the concentration of reducing sugars prepared by polysaccharide hydrolysis in plant milk and in plant cheese, and moisture content of plant cheese was performed by one-way analysis of variance (ANOVA) using IBM SPSS statistics 23. *P* value of ≤0.05 with Tukey adjustment for multiple comparisons was considered statistically significant.

## 5.3 Results

### 5.3.1 Inhibition of microbial growth during germination by chlorine and / or LAB

The soaking and germination of seeds supports the growth of undesirable microorganisms from the plant or the environment (Taormina et al., 1999). In this study, it was found that the germination of lupine seeds was not influenced by 2,000 ppm of chlorine or LAB (data not shown). Cell counts of raw lupine seeds on LB, M17 and MRS agars were about $10^5$ cfu/g (Table 5.2). Colonies on M17 and MRS agars were identified as *Pediococcus* spp. and *Weissella* spp. by colony PCR to amplify 16S rRNA genes using primers (forward: 5'-AGAGTTTGATCCTGGCTCAG-3'; reverse: 5'-GGCTACCTTGTTACGACTT-3'; data not shown). Cell counts on VRBG agar were below 10 cfu/mL. Chlorine treatment did not reduce the cell counts. During seed germination, cell counts on LB and M17 agars increased about 100 and 10-fold, respectively, and counts on VRBG increased to about $10^5$ cfu/g. Chlorine treatment or addition of *Lc. lactis* and *Lp. plantarum* prevented the increase of cell counts on LB and VRBG agars; addition of LAB resulted in cell counts of *Lc. lactis* and *Lp. plantarum* with about $10^8$ and $10^9$ cfu/g after germination (Table 5.2).

**Table 5.2.** Cell counts of lupine seeds based on selective agar plates. Lupine seeds were ungerminated; pre-treated with chlorine solution and ungerminated; germinated without lactic acid bacteria (LAB, *Lp. plantarum* and *Lc. lactis*); pre-treated with chlorine solution, then germinated without *Lp. plantarum* and *Lc. lactis*; germinated with *Lp. plantarum* and *Lc. lactis*; pre-treated with chlorine solution, then germinated with *Lp. plantarum* and *Lc. lactis*.

| | Cell counts [log(cfu/g)] | | | |
|---|---|---|---|---|
| **Samples** | **LB agar** | **M17 agar** | **MRS agar** | **VRBG agar** |
| no chlorine, ungerminated | 5.3 ± 0.3 | 5.5 ± 0.3 | 5.4 ± 0.4 | < 1 |
| chlorine, ungerminated, | 5.5 ± 0.4 | 5.6 ± 0.2 | 5.6 ± 0.3 | |
| no chlorine, germinated | 7.3 ± 0.4 | 6.7 ± 1.1 | <6.0 | 4.7 ± 0.1 |
| chlorine, germinated | 5.6 ± 0.5 | 5.2 ± 0.9 | | |
| no chlorine, germinated with LAB | 5.5 ± 0.5 | 7.9 ± 0.1 | 8.7 ± 0.2 | <4.0 |
| chlorine, germinated with LAB | 5.3 ± 0.9 | 7.6 ± 0.1 | 8.4 ± 0.2 | |

Sequencing revealed that alpha-proteobacteria and cyanobacteria were the most abundant phyla on ungerminated seeds (Table 5.3). The relative abundance of cyanobacteria decreased after chlorine treatment and the relative abundance of all other taxa increased (Table 5.3). The growth of *Enterobacteriaceae* was inhibited by chlorine or LAB, matching the sequencing result. During germination, the relative abundance of gamma-proteobacteria and *Bacillota* increased while the relative abundance of all taxa except cyanobacteria remained unchanged during germination of chlorine treated cells. During germination in presence of *Lc. lactis* and *Lp. plantarum*, *Lactobacillales* accounted for more than 90% of all 16S rRNA genes (Table 5.3). All of these sequences were more than 99.5% identical to the 16S rRNA gene sequences of *Lc. lactis* or *Lp. plantarum* (data not shown) while *Pediococcus* and *Weissella* were the most abundant LAB on seeds germinated without addition of protective cultures.

**Table 5.3.** Relative abundance (%) of bacteria identified in ungerminated and germinated lupin seeds as determined by high throughput sequencing of full length 16S rRNA genes.

| Samples | proteobacteria | | Cyanophyceae | *Bacillota* | *Lactobacillales* | # of reads (k) |
|---|---|---|---|---|---|---|
| | Alpha- | Gamma- | | | | |
| no chlorine, ungerminated | 58.7 ± 7.4 | 2.0 ± 0.3 | 32.6 ± 10.8 | 5.4 ± 3.5 | 5.1 ± 3.5 | 8.9 – 14.1 |
| chlorine, ungerminated, | 72.7 ± 14.0 | 2.3 ± 0.4 | 13.7 ± 10.2 | 9.5 ± 4.7 | 9.1 ± 4.7 | 6.4 – 10.5 |
| no chlorine, germinated | 28.2 ± 4.2 | 34.9 ± 28.1 | 2.4 ± 1.3 | 33.7 ± 24.3 | 1.5 ± 1.8 | 1.0 – 7.8 |
| chlorine, germinated | 84.7 ± 6.7 | 4.1 ± 1.0 | 2.3 ± 0.4 | 6.7 ± 6.7 | 6.2 ± 6.7 | 0.5 – 7.0 |
| no chlorine, germinated with LAB | 1.5 ± 0.5 | 0.1 ± 0.0 | 0.6 ± 0.2 | 97.8 ± 0.7 | 97.7 ±0.7 | 5.6 – 15.5 |
| chlorine, germinated with LAB | 5.0 ± 2.3 | 0.2 ± 0.1 | 1.5 ± 1.2 | 93.3 ± 3.5 | 93.3 ± 3.5 | 9.2 – 20.9 |

### 5.3.2 Acidification of plant milk

In a preliminary screening, the acidification of soymilk by 9 strains of LAB was compared (Table 5.4). These species were selected to include lactobacilli that frequently occur in fermented plant foods (Gänzle, 2022). The result of acidification kinetics of LAB to soymilk showed that *Lp. plantarum* acidified plant milk faster than all other LAB; the pH value after 12 h of fermentation with *Lp. plantarum* was 3.86 ± 0.09 while other LAB acidified to pH values ranging from 4.16 – 4.37 (Table 5.4).

**Table 5.4.** Acidification kinetics of different lactic acid bacteria to soymilk.

| Strains | pH | | |
|---|---|---|---|
| | 0 h | 6 h | 12 h |
| *Lactococcus lactis* FUA3228 | 6.49 ± 0.05 | 5.10 ± 0.31 | 4.37 ± 0.15 |
| *Lactiplantibacillus plantarum* TMW1.460 | | 4.58 ± 0.08 | 3.86 ± 0.09 |
| *Lacticaseibacillus paracasei* FUA3413 | | 5.20 ± 0.42 | 4.16 ± 0.11 |
| *Levilactobacillus hammesii* TMW1.1236 | | 5.34 ± 0.53 | 4.29 ± 0.10 |
| *Furfurilactobacillus milli* FUA3430 | | 5.32 ± 0.48 | 4.33 ± 0.11 |
| *Limosilactobacillus reuteri* TMW1.656 | | 5.34 ± 0.50 | 4.26 ± 0.09 |
| *Lentilactobacillus buchneri* FUA3252 | | 5.40 ± 0.54 | 4.30 ± 0.03 |
| *Weissella cibaria* 10M | | 5.08 ± 0.28 | 4.31 ± 0.10 |
| *Leuconostoc mesenteroides* FUA3090 | | 4.70 ± 0.08 | 4.22 ± 0.22 |

*Lp. plantarum* and *Lc. lactis* were selected to control plant microbiota during soaking and germination of plant seeds, and to subsequently act as primary acidifying cultures. *Bacillus* spp. are major fermentation microorganisms for many traditional food fermentation of legumes, such as *natto*, *sufu*, *cheonggukjang*, and *hawaijar* (Li et al., 2023b). They were thus introduced into the fermentation of plant cheese at the stage of soaking and germination to investigate whether their hydrolytic enzymes impact cheese quality. The initial cell counts of *Lp. plantarum*, *Lc. lactis*, or *Bacillus* spp. in soy or lupine milk from germinated seeds were consistent with those in soaking cultures, ranging from $10^7 – 10^8$ cfu/ml (Table 5.1). In ungerminated milk, the initial cell counts of starter cultures were thus adjusted to the same level. After 12 h of acidification, pH values of all plant milk samples were ≤ 4.0 (Table 5.5). The rate of plant milk acidification was not influenced by *Bacillus* spp.

**Table 5.5.** pH of soymilk and lupin milk before and after 12 h of fermentation. Plant milk were made from ungerminated seeds or seeds germinated: with LAB only, or LAB with *B. velezensis* or with *B. amyloliquefaciens*.

| Samples | pH value of soymilk | | pH value of lupin milk | |
|---|---|---|---|---|
| | 0 h | 12 h | 0 h | 12 h |
| ungerminated | 6.50 ± 0.01 | 3.90 ± 0.11 | 5.65 ± 0.05 | 3.6 ± 0.10 |
| germinated with LAB | 6.42 ± 0.01 | 3.83 ± 0.03 | 5.97 ± 0.09 | 3.7 ± 0.20 |
| germinated with LAB and *B. velezensis* | 6.54 ± 0.12 | 3.93 ± 0.04 | 5.90 ± 0.04 | 4.1 ± 0.40 |
| germinated with LAB and *B. amyloliquefaciens* | 5.75 ± 0.14 | 4.00 ± 0.05 | -- | -- |

### 5.3.3 Quantification of carbohydrates in plant milks

To assay the impact on carbohydrates of seeds of germination and *Bacillus* spp., galactose, glucose, fructose, sucrose, melibiose, raffinose, stachyose, and verbascose were quantified in plant milk from ungerminated seeds or seeds germinated with or without *Bacillus* spp.(Figure 5.1). Sucrose was the main sugar in plant milk from both germinated and ungerminated soy or lupine seeds. Compared to the plant milk from ungerminated seeds, plant milk from germinated seeds had lower amounts of mono-, di-, and oligosaccharides, indicating these sugars were consumed during germination (Figure 5.1). Soymilk from seeds germinated with LAB and *Bacillus* spp. contained higher amounts of sucrose than that from seeds germinated with LAB only. Soymilk had higher amounts of glucose and fructose but contained lower amounts of galactose, and verbascose than lupine milk (Figure 5.1). Also, they exhibited differences in the consumption of carbohydrates during fermentation. Sucrose was the main carbon source of starter cultures in soymilk acidification; after 12 h of fermentation, it was depleted in soymilk from seeds germinated with LAB and *B. amyloliquefaciens* (Figure 5.1). Glucose and fructose were consumed during acidification of soymilk from germinated seeds rather than soymilk from ungerminated seeds over

12 h of fermentation. However, concentration of sucrose, glucose, and fructose in lupine milk did not decrease after fermentation.

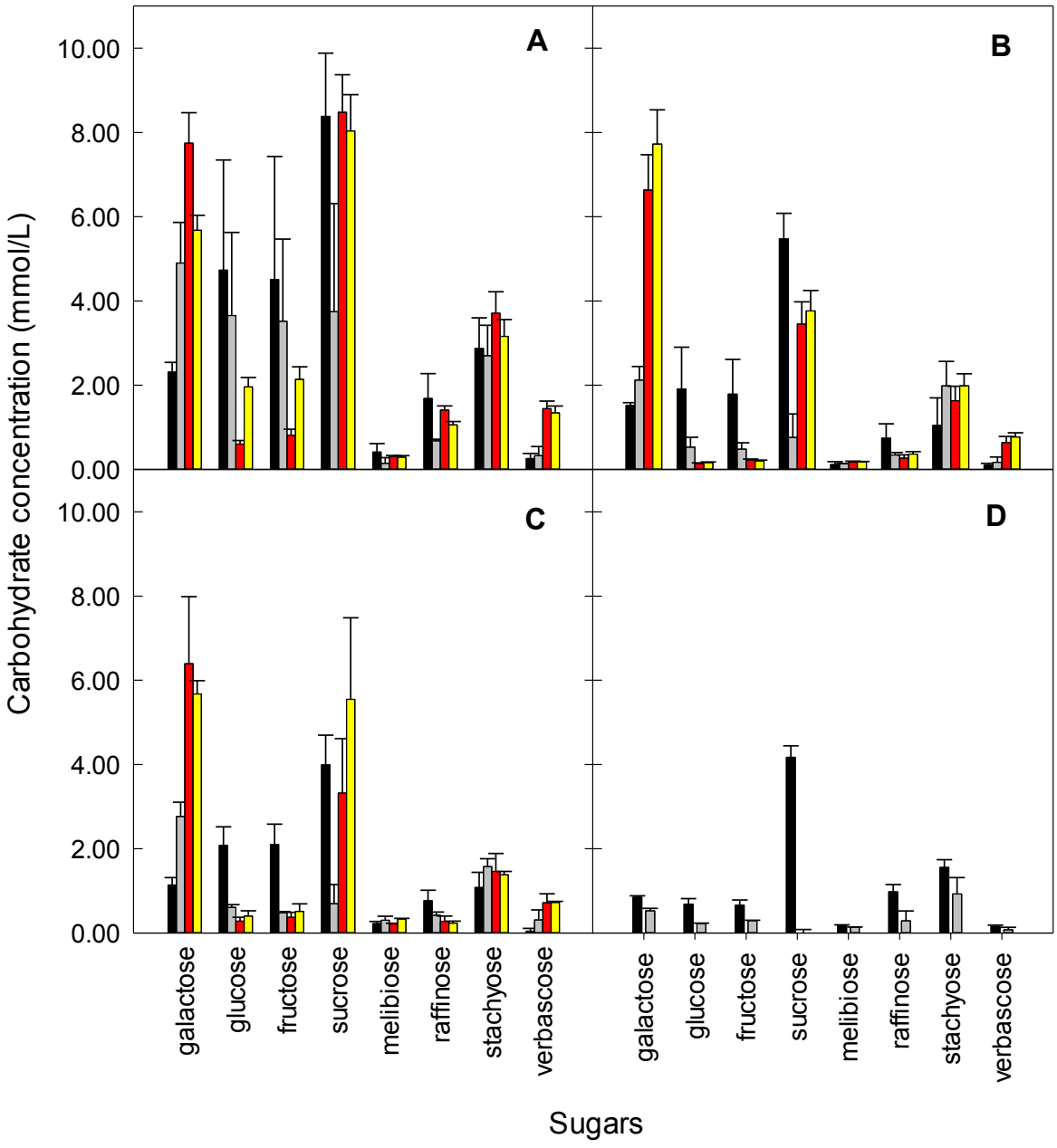



**Figure 5.1.** Carbohydrate concentrations in plant milk from ungerminated seeds (A) or seeds germinated with: B) LAB only, C) LAB and *B. velezensis*, or D) LAB and *B. amyloliquefaciens*. Colours indicate the carbohydrate concentrations in unfermented (black) soymilk and soymilk after 12 h of fermentation (grey), and in unfermented lupin milk (red) and lupine milk after 12 h of fermentation.

### 5.3.4 The effect of germination and *Bacillus* spp. on the growth of starter cultures during the acidification and ripening of plant cheeses

The cell counts of inocula during cheese ripening were monitored after surface plating and observation of the morphology of colonies on agar plates. The colony morphology of all colonies observed from all samples matched the colony morphology of one of the three strains that was used as inoculum. The sequence of 97.2% or more of 16S rRNA genes amplified from community DNA of soy and lupine cheese samples matched the 16S rRNA genes sequences of one of the three strains used as inoculum with a sequence identity of more than 99.5% (Table 5.6 and Table 5.7), confirming that cheese microbiota were fully controlled by the two or three strain inocula.

**Table 5.6.** Relative abundance (%) of starter cultures in soy cheeses ripened for 0, 14, 45, and 90 days as determined by high throughput sequencing of full length 16S rRNA genes.

| Samples | *Lp. plantarum* | *Lc. lactis* | *Bacillus* spp. | Total | # of reads (k) |
|---|---|---|---|---|---|
| | 0 d | | | | |
| ungerminated | 54.5 ± 5.4 | 44.6 ± 5.4 | 0 | 99.1 ± 0.1 | 7.9 – 9.0 |
| germinated with LAB | 54.4 ± 3.2 | 44.7 ± 3.5 | 0 | 99.1 ± 0.3 | 7.4 – 13.2 |
| germinated with LAB and *B. velezensis* | 66.2 ± 5.3 | 32.4 ± 5.1 | 0 | 98.7 ± 0.4 | 2.5 – 20.3 |
| germinated with LAB and *B. amyloliquefaciens* | 72.8 ± 8.3 | 23.1 ± 10.0 | 1.2 ± 1.6 | 97.2 ± 1.5 | 8.2 – 13.6 |
| | 14 d | | | | |
| ungerminated | 52.7 ± 5.7 | 46.6 ± 5.6 | 0 | 99.3 ± 0.2 | 2.3 – 4.1 |
| germinated with LAB | 53.1 ± 10.4 | 46.1 ± 10.6 | 0 | 99.2 ± 0.2 | 4.4 – 6.0 |
| germinated with LAB and *B. velezensis* | 69.3 ± 4.2 | 30.0 ± 4.3 | 0 | 99.3 ± 0.1 | 6.7 – 8.8 |
| germinated with LAB and *B. amyloliquefaciens* | 75.1 ± 9.1 | 23.1 ± 9.5 | 0.4 ± 0.6 | 98.7 ± 0.4 | 7.7 – 15.7 |
| | 45 d | | | | |
| ungerminated | 53.3 ± 1.8 | 46.1 ± 1.6 | 0 | 99.4 ± 0.2 | 15.7 – 19.6 |
| germinated with LAB | 65.4 ± 11.8 | 33.9 ± 11.8 | 0 | 99.2 ± 0.3 | 1.2 – 12.1 |
| germinated with LAB and *B. velezensis* | 69.5 ± 3.4 | 29.9 ± 3.4 | 0 | 99.4 ± 0.0 | 7.5 – 13.2 |
| germinated with LAB and *B. amyloliquefaciens* | 75.2 ± 4.0 | 22.3 ± 2.6 | 0.3 ± 0.2 | 97.8 ± 1.6 | 6.0 – 10.5 |
| | 90 d | | | | |
| ungerminated | 25.0 ± 2.5 | 74.6 ± 2.2 | 0 | 99.5 ± 0.3 | 6.2 – 13.5 |
| germinated with LAB | 69.2 ± 9.5 | 30.3 ± 9.7 | 0 | 99.6 ± 0.2 | 1.4 – 9.0 |
| germinated with LAB and *B. velezensis* | 67.4 ± 9.5 | 31.9 ± 9.5 | 0 | 99.3 ± 0.2 | 5.5 – 6.9 |
| germinated with LAB and *B. amyloliquefaciens* | 80.1 ± 6.4 | 18.5 ± 6.1 | 0.3 ± 0.3 | 98.9 ± 0.3 | 10.5 – 11.9 |

**Table 5.7.** Relative abundance (%) of starter cultures in lupine cheeses ripened for 0, 14, 45, and 90 days as determined by high throughput sequencing of full length 16S rRNA genes.

| Samples | *Lp. plantarum* | *Lc. lactis* | *Bacillus* spp. | Total | # of reads (k) |
|---|---|---|---|---|---|
| | 0 d | | | | |
| ungerminated | 83.8 ± 5.3 | 15.8 ± 5.2 | 0 | 99.6 ± 0.1 | 4.9 – 30.2 |
| germinated with LAB | 97.6 ± 0.7 | 1.6 ± 1.0 | 0 | 99.2 ± 0.3 | 2.4 – 8.6 |
| germinated with LAB and *B. velezensis* | 60.5 ± 18.0 | 37.0 ± 20.9 | 0 | 97.4 ± 3.3 | 5.9 – 13.2 |
| | 14 d | | | | |
| ungerminated | 88.3 ± 5.5 | 10.8 ± 5.8 | 0 | 99.1 ± 0.9 | 13.2 – 19.4 |
| germinated with LAB | 98.1 ± 0.8 | 1.3 ± 0.8 | 0 | 99.4 ± 0.0 | 7.6 – 11.2 |
| germinated with LAB and *B. velezensis* | 59.0 ± 21.5 | 39.8 ± 22.4 | 0 | 98.8 ± 1.1 | 7.1 – 22.4 |
| | 45 d | | | | |
| ungerminated | 85.9 ± 4.4 | 13.8 ± 4.4 | 0 | 99.7 ± 0.1 | 15.0 – 39.0 |
| germinated with LAB | 97.7 ± 1.2 | 1.8 ± 1.1 | 0 | 99.6 ± 0.1 | 0.4 – 9.5 |
| germinated with LAB and *B. velezensis* | 58.0 ± 18.1 | 41.4 ± 18.2 | 0 | 99.4 ± 0.3 | 0.7 – 68.5 |
| | 90 d | | | | |
| ungerminated | 82.4 ± 4.0 | 17.3 ± 4.1 | 0 | 99.7 ± 0.1 | 5.3 – 54.0 |
| germinated with LAB | 97.3 ± 1.6 | 2.2 ± 1.8 | 0 | 99.5 ± 0.2 | 0.7 – 5.8 |
| germinated with LAB and *B. velezensis* | 51.6 ± 14.7 | 47.8 ± 14.7 | N/A | 99.5 ± 0.2 | 7.7 – 16.6 |

The cell counts of *Lp. plantarum* and *Lc. lactis* in unripened plant cheeses were 10 – 11 log and 8 – 10 log (cfu/g), respectively, showing two strains as dominant cultures during plant milk acidification (Figure 5.2 and Figure 5.3). In contrast, cell counts of *B. velezensis* were ~ $10^8$ cfu/g in unripened soy cheeses and ~$10^7$ cfu / g in unripened lupine cheeses, respectively, which were the same to those in fresh plant milk, indicating that *B. velezensis* was unchanged during milk acidification (Figure 5.2); *B. amyloliquefaciens* had about one log of reduction after acidification (Figure 5.2). During cheese ripening, cell counts of bacilli remained unchanged (Figure 5.2 and Figure 5.3). Cell counts of *Lp. plantarum* decreased by about 1 log (cfu/g) in soy cheese and by

about 2 log (cfu/g) in lupine cheese; this decrease was not impacted by either germination or by bacilli (Figure 5.2 and Figure 5.3). Cell counts of *Lc. lactis* decreased to levels below the detection limit during ripening. The presence of bacilli during seed germination substantially delayed the inactivation of *Lc. lactis* during cheese ripening (Figure 5.2 and Figure 5.3).

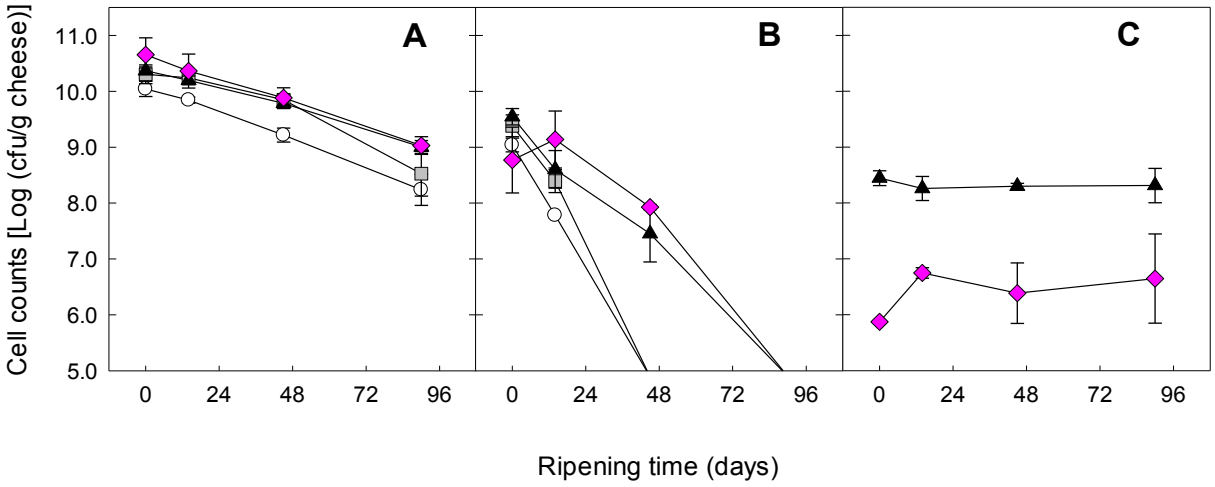



**Figure 5.2.** Cell counts of A) *Lp. plantarum*, B) *Lc. lactis*, and C) *Bacillus* spp. in soy cheeses ripened for 0, 14, 45, and 90 d. Symbols indicate the cell counts for soy cheese from ungerminated seeds (open circle) or from seeds germinated: with LAB only (grey square), LAB with *B. velezensis* (black triangle up), or LAB with *B. amyloliquefaciens* (pink diamond). The y-axis is scaled to indicate the detection limit of $10^5$ cfu/g and lines dropping below the x-axis indicate values below the detection limit.

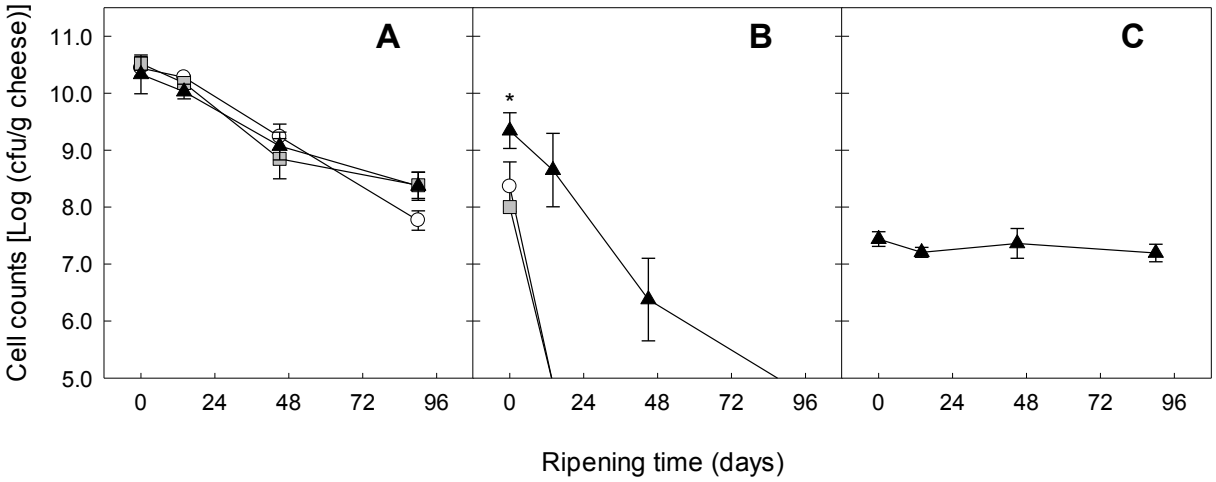



**Figure 5.3.** Cell counts of A) *Lp. plantarum*, B) *Lc. lactis*, and C) *B. velezensis* in lupin cheeses ripened for 0, 14, 45, and 90 d. Symbols indicate the cell counts for lupin cheese from ungerminated seeds (open circle) or from seeds germinated: with LAB only (grey square), or LAB with *B. velezensis* (black triangle up). The y-axis is scaled to indicate the detection limit of $10^5$ cfu/g and lines dropping below the x-axis indicate values below the detection limit.

### 5.3.5 Characterization of mono-, di-, oligo-, and polysaccharides in ripened plant cheeses

The metabolism of mono-, di-, oligo-, and polysaccharides during cheese ripening was characterized. The carbohydrate concentration in cheese samples was lower than that in milk samples, indicating that most of carbohydrates were retained in the supernatants after centrifugation, then removed. Soy cheeses from ungerminated seeds had higher amounts of carbohydrates relative to soy cheese from germinated seeds (Figure 5.4), but no differences in the content of mono-, di-, and oligosaccharides were observed between lupine cheeses from ungerminated seeds and from germinated seeds (Figure 5.5).

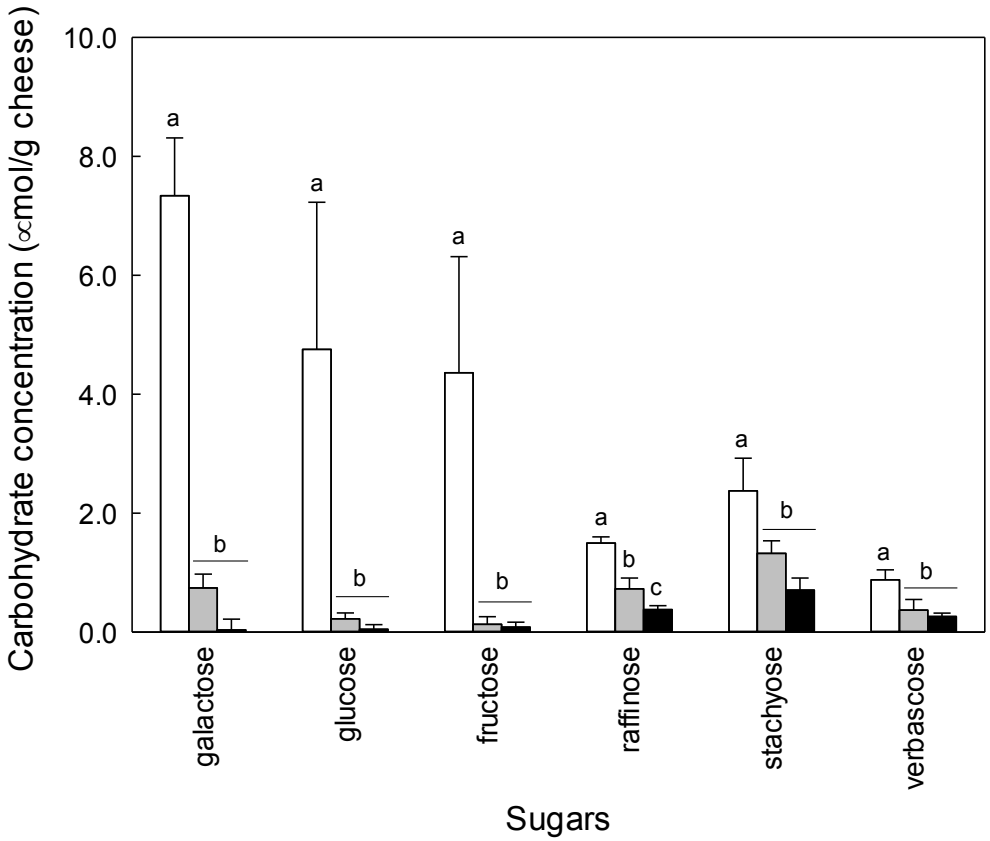



**Figure 5.4.** Carbohydrate concentration in unripened soy cheeses. Colours indicate carbohydrate concentration in unripened soy cheese from ungerminated seeds (white) or from seeds germinated: with LAB only (grey), or LAB with *B. velezensis* (black).

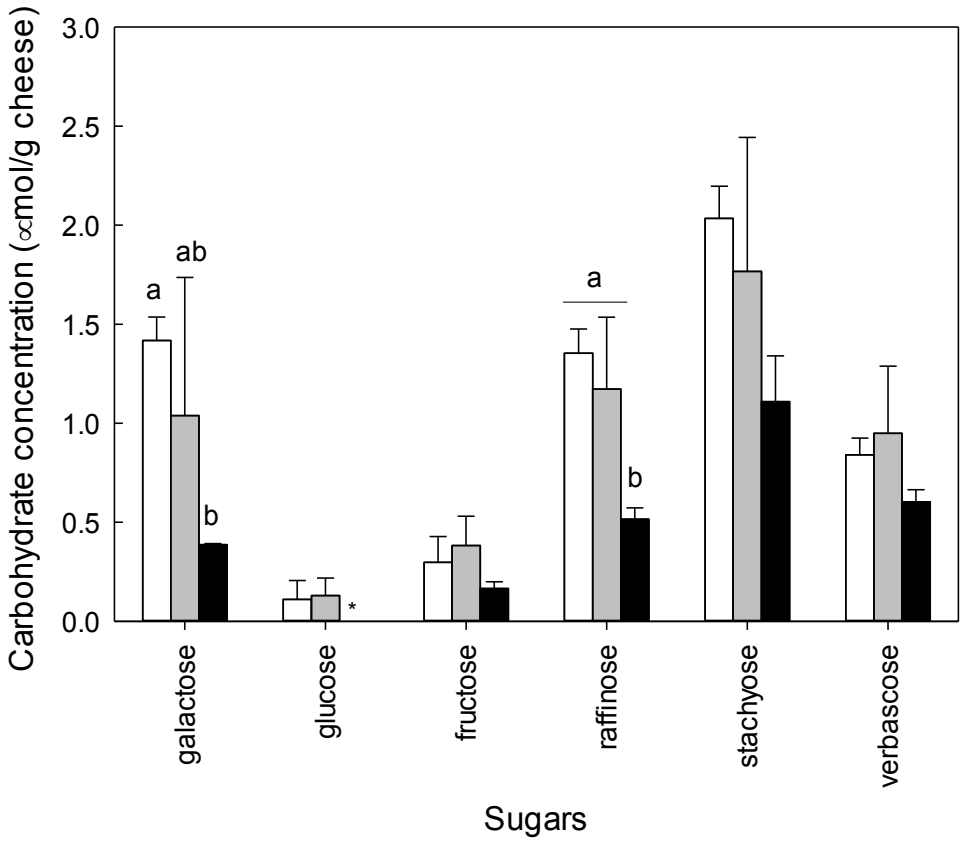



**Figure 5.5.** Carbohydrate concentration in unripened lupin cheeses. Colours indicate carbohydrate concentration in lupin cheese from ungerminated seeds (white) or from seeds germinated: with LAB only (grey), or LAB with *B. velezensis* (black).

The main carbon source in initial stages of ripening was oligosaccharides including raffinose, stachyose, and verbascose, which were completely consumed within 2 weeks of ripening (Figure 5.6 and Figure 5.7). In plant cheeses from ungerminated seeds and in plant cheeses that were made from seeds germinated with LAB only, the resulting galactose, glucose, fructose, or sucrose was also depleted within 2 weeks of ripening. Two of unknown oligosaccharides were detected in soy and lupine cheeses and they were labeled with peak 4 and 7 in Figure 5.6 and Figure 5.7. The plant cheeses from seeds germinated with the addition of *Bacillus* spp. had higher

amounts of an unknown oligosaccharide that was labeled with peak 7, compared to plant cheeses from ungerminated seeds or plant cheeses from seeds germinated with LAB only.

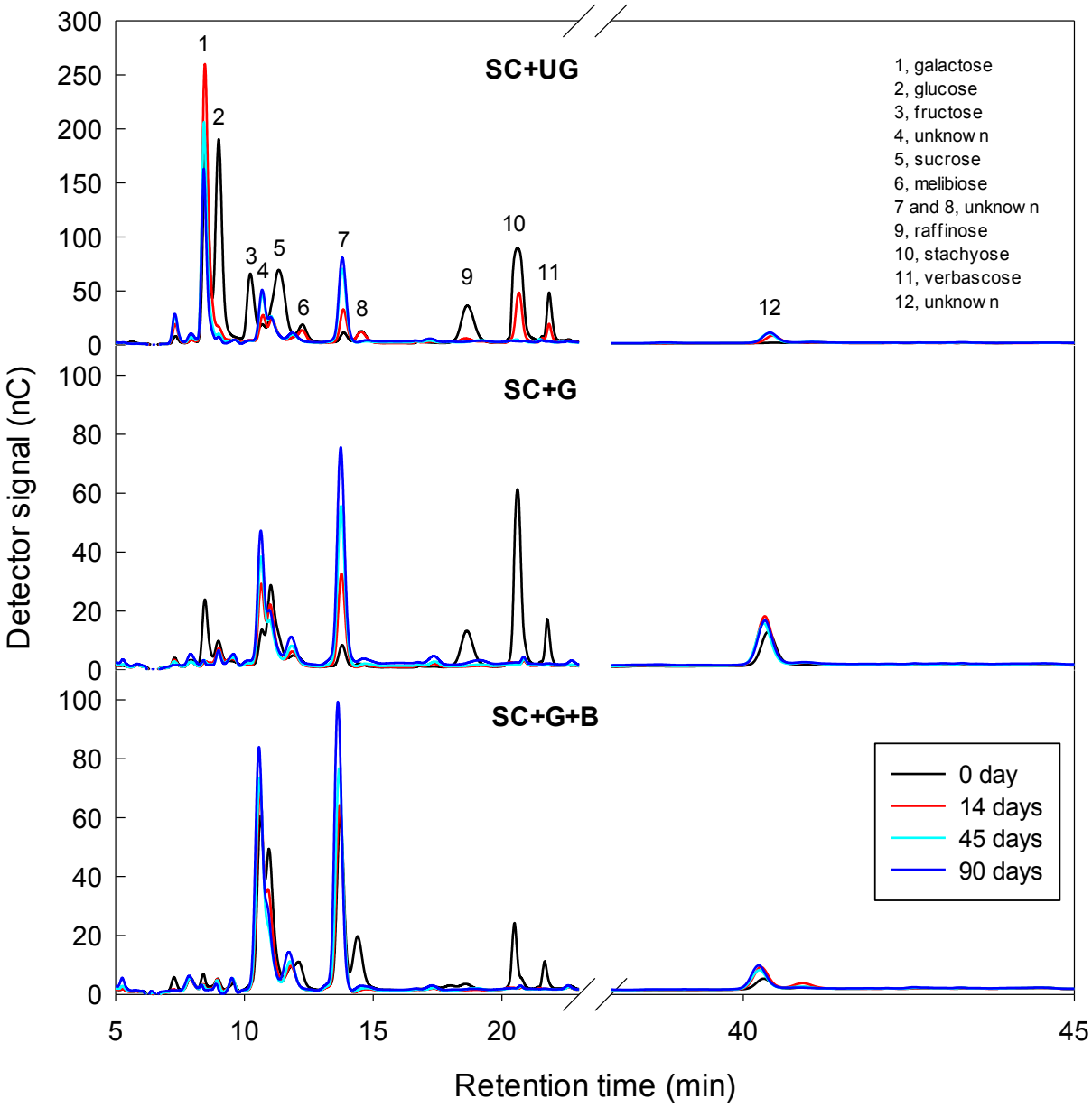



**Figure 5.6.** Chromatograms of carbohydrates in soy cheeses ripened for 0, 14, 45, and 90 days. Colours indicate soy cheese ripened for 0 (black), 14 (red), 45 (cyan), and 90 (blue) days. Soy cheese was produced from ungerminated seeds (SC+UG) or from seeds germinated: with LAB only (SC+G), or LAB with *B. velezensis* (SC+G+B).

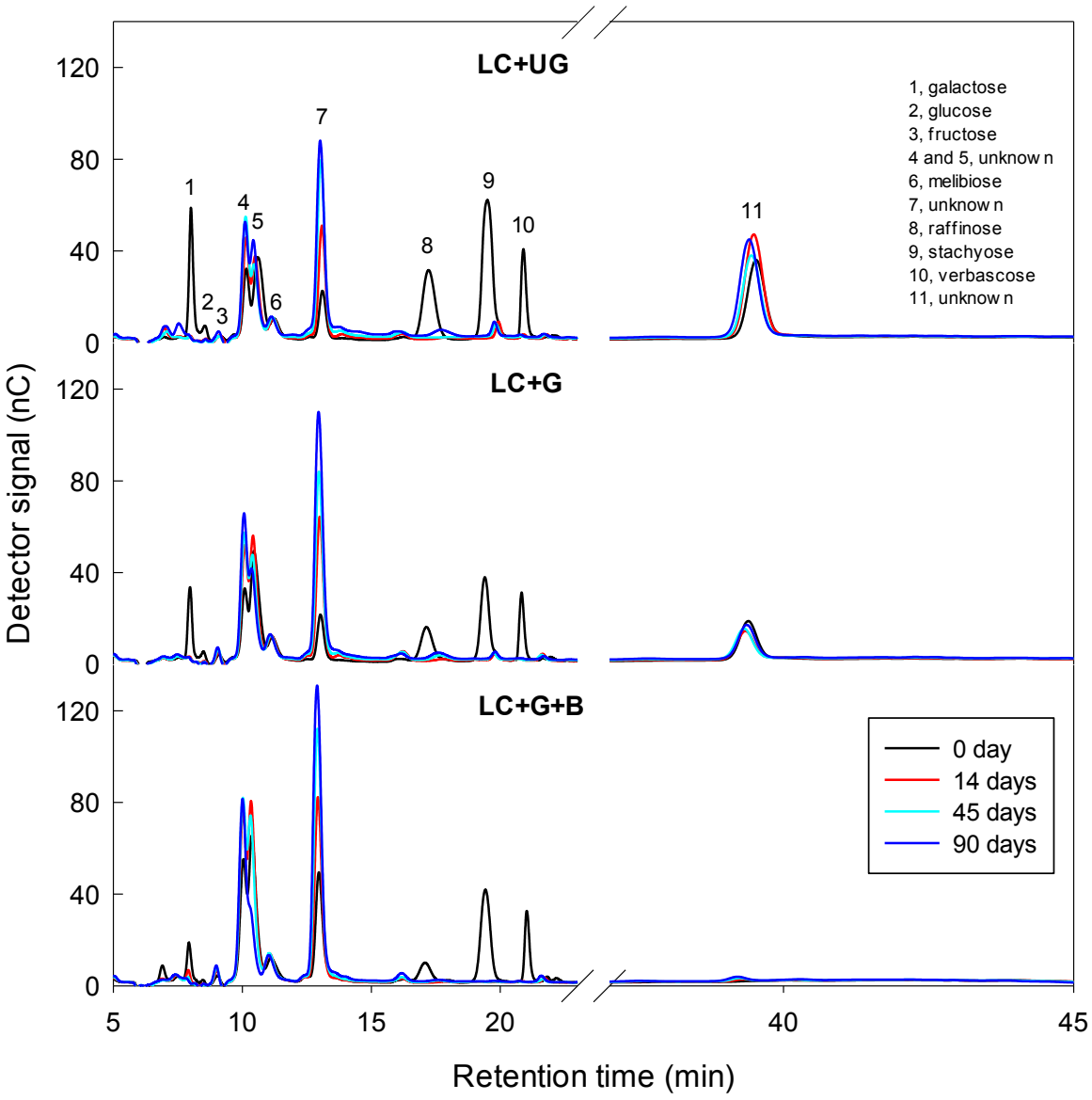



**Figure 5.7.** Chromatograms of carbohydrates in lupin cheeses ripened for 0, 14, 45, and 90 days. Colours indicate lupin cheese ripened for 0 (black), 14 (red), 45 (cyan), and 90 days (blue). Lupin cheese was produced from ungerminated seeds (LC+UG) or from germinated: with LAB only (LC+G), or LAB with *B. velezensis* (LC+G+B).

The pH and the organic acid concentrations in 14 d ripened plant cheeses reflected the sugar concentration, i.e. lactate and acetate concentrations where highest in plant cheeses from ungerminated seeds, followed by plant cheeses from germinated seeds and plant cheeses from

germinated seeds produced with bacilli (Table S5.1 and Table S5.2). During cheese ripening, the pH slightly increased, lactate concentrations decreased, and acetate concentrations increased, likely indicating conversion of lactate to acetate via pyruvate dehydrogenase (Gänzle, 2015).

The concentration of water-soluble polysaccharides was higher in plant milk from ungerminated seeds when compared to plant milk from germinated seeds (Table 5.8). Addition of bacilli during seed germination increased the concentration of water-soluble polysaccharides in soymilk but not in lupine milk (Table 5.8). During cheese ripening, the concentration of water-soluble polysaccharides increased in all samples except the samples produced from soy seeds that were germinated in presence of bacilli (Table 5.8). In summary, both germination and the presence of bacilli during germination impact the concentration of sugars and of water-soluble polysaccharides in plant cheeses and their evolution during cheese ripening.

**Table 5.8.** The concentration of reducing sugars prepared by hydrolysis of water-soluble polysaccharides in plant milk and in cheeses that were made from ungerminated or germinated seeds. Cheeses were fermented with LAB only, or LAB with *B. velezensis*.

| **Samples** | **Plant milk (mmol/L)** | | **Soy cheese (µmol/g dry weight)** | |
|---|---|---|---|---|
| | soymilk | lupine milk | 0 d | 90 d |
| ungerminated | $2.41 \pm 0.19^{a}$ | $7.96 \pm 1.16^{a}$ | $4.57 \pm 0.50^{a}$ | $6.22 \pm 1.22^{a*}$ |
| germinated with LAB | $1.58 \pm 0.33^{b}$ | $5.93 \pm 0.48^{b}$ | $2.31 \pm 0.55^{c}$ | $3.90 \pm 0.54^{b*}$ |
| germinated with LAB and *B. velezensis* | $2.47 \pm 0.34^{a}$ | $5.72 \pm 0.13^{b}$ | $3.50 \pm 0.21^{b}$ | $2.84 \pm 0.70^{b}$ |

Values in the same column that do not share a lowercase superscript differ significantly ($P<0.05$). Significant differences ($P<0.05$) between ripened and unripened soy cheeses are indicated with an asterisk.

### 5.3.6 Changes in the concentrations of free amino nitrogen and moisture contents of ripened plant cheeses

Proteolysis during cheese ripening was monitored by quantification of free amino nitrogen and by qualitative separation of proteins isolated from cheese samples by SDS-PAGE. The concentration of free amino nitrogen increased during ripening of both soy and lupine cheeses (Figure 5.8). Seed germination did not impact the concentration of free amino nitrogen (Figure 5.8). In soy and lupine cheeses that were made from seeds that were germinated with *Bacillus* spp., the concentration of free amino nitrogen was higher than in samples from seeds germinated with LAB only. The difference was greater in soy cheeses than in lupine cheeses.

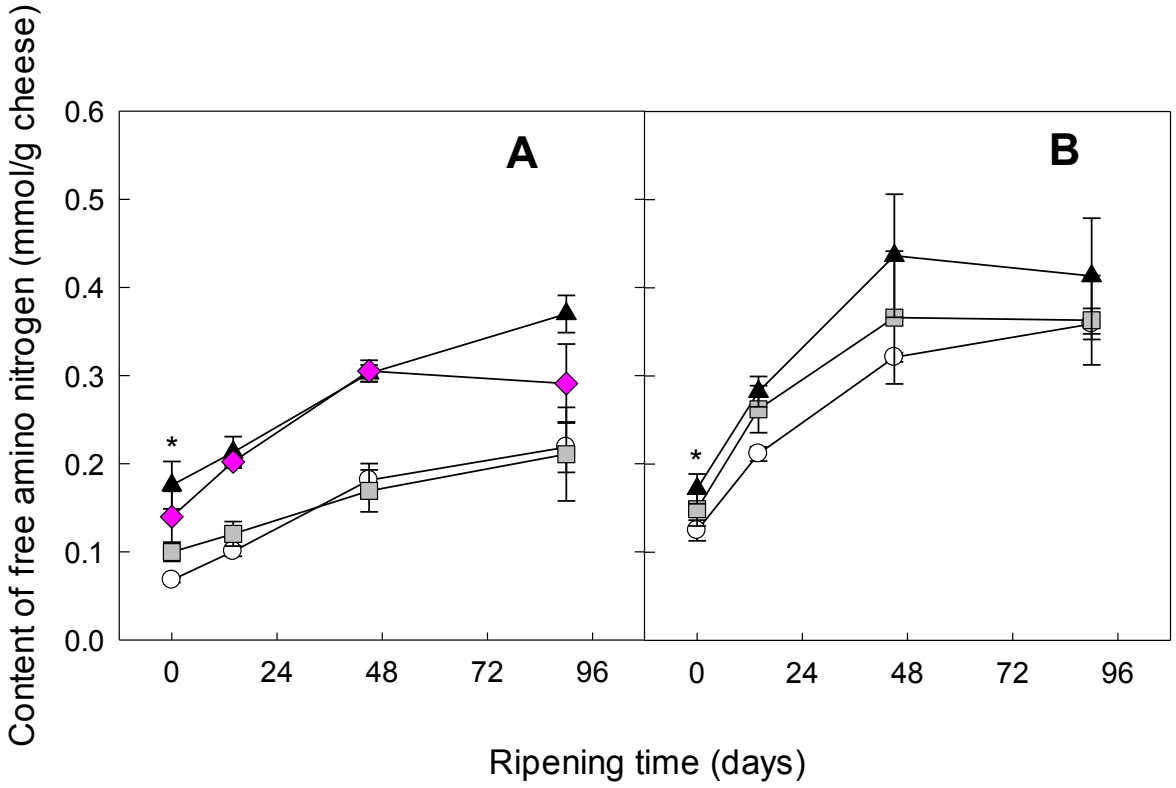



**Figure 5.8.** Free amino nitrogen (FAN) contents in soy (A) and lupin (B) cheeses ripened for 0, 14, 45, and 90 d. Symbols indicate FAN content in cheese from ungerminated seeds (open circle), or from seeds germinated: LAB only (grey square), or LAB with *B. velezensis* (black triangle up) or with *B. amyloliquefaciens* (pink diamond).

SDS-PAGE analysis of proteins in cheese samples documented that the intensity of protein bands was largely unchanged in samples produced from ungerminated seeds, or from seeds that were germinated with LAB only. In cheese samples that were made from soy beans germinated with LAB and bacilli, several high molecular weight proteins decreased during ripening while several protein bands with a low molecular weight became apparent (Figure 5.9). In cheese samples that were produced from either germinated or ungerminated lupine seeds with or without addition of bacilli, protein bands remained largely unchanged during ripening (Figure 5.9).

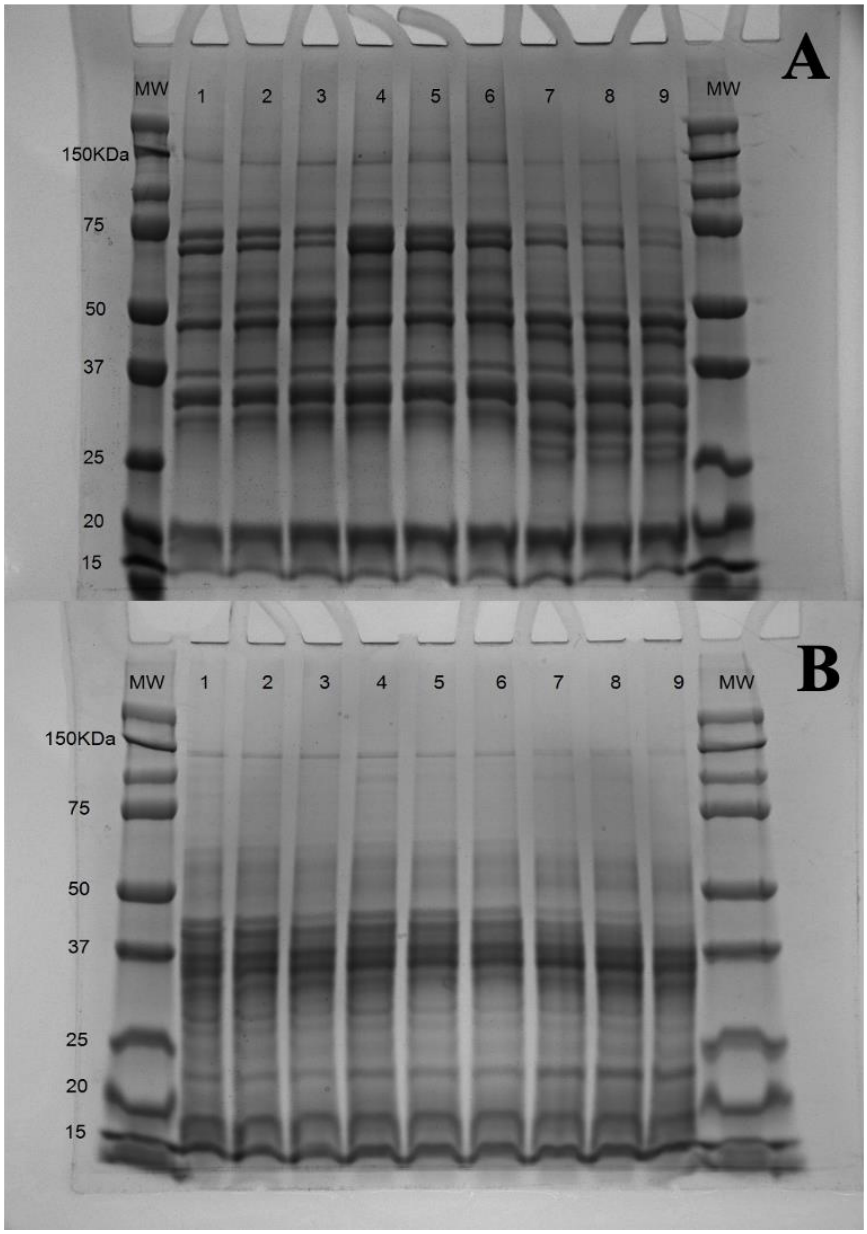



**Figure 5.9.** SDS-PAGE of proteins in soy (A) and lupin (B) cheeses ripened for 0, 14, and 45 days. Lane 1 and 11, molecular weight marker; lane 2 to 4, plant cheese from ungerminated seeds ripened for 0, 14, and 45 days, respectively; lane 5 to 7, plant cheese from seeds germinated with LAB only ripened for 0, 14, and 45 days, respectively; lane 8 to 10, plant cheese from seeds germinated with LAB and *B. velezensis* and ripened for 0, 14, and 45 days, respectively.

The moisture content of soy cheese from ungermianted seeds was higher than that of soy cheese from germinated seeds. Significant ($P$<0.05) differences in the moisture content were not observed between lupine cheeses from ungerminated seeds and from seeds germinated with LAB

only, but their moisture contents were higher than that of lupine cheese from seeds germinated with *Bacillus* spp. The moisture content was reduced at least 10% for each plant cheese after 90 days of ripening relative to unripened plant cheese (Table 5.9).

**Table 5.9.** Moisture content (%) of soy and lupin cheeses during ripening from 0, 14, 45, and 90 days. Plant cheeses were made from ungerminated seeds or seeds germinated: with LAB only, or LAB with *B. velezensis* or with *B. amyloliquefaciens*.

| | **Moisture content (%)** | | | |
|---|---|---|---|---|
| **Samples** | **0 d** | **14 d** | **45 d** | **90 d** |
| Soy cheese | | | | |
| ungerminated | $77 \pm 3^{a}$ | $74 \pm 2^{a}$ | $72 \pm 3^{a}$ | $67 \pm 4^{a*}$ |
| germinated with LAB | $66 \pm 3^{b}$ | $59 \pm 2^{bc}$ | $54 \pm 3^{c}$ | $50 \pm 2^{b*}$ |
| germinated with LAB and *B. velezensis* | $59 \pm 1^{c}$ | $55 \pm 1^{c}$ | $53 \pm 2^{c}$ | $45 \pm 1^{c*}$ |
| germinated with LAB and *B. amyloliquefaciens* | $66 \pm 2^{b}$ | $61 \pm 2^{b}$ | $60 \pm 2^{b}$ | $51 \pm 3^{b*}$ |
| Lupine cheese | | | | |
| ungerminated | $69 \pm 3^{a}$ | $66 \pm 3^{a}$ | $63 \pm 2^{a}$ | $59 \pm 2^{a*}$ |
| germinated with LAB | $73 \pm 4^{a}$ | $66 \pm 4^{a}$ | $64 \pm 1^{a}$ | $58 \pm 5^{a*}$ |
| germinated with LAB and *B. velezensis* | $61 \pm 1^{b}$ | $57 \pm 1^{b}$ | $52 \pm 2^{b}$ | $36 \pm 8^{b*}$ |

[*] Values in the same column that do not share a lowercase superscript differ significantly ($P<0.05$). Significant differences ($P<0.05$) between ripened for 90 d and unripened soy or lupine cheeses are indicated with an asterisk.

## 5.4 Discussion

We used a combination of culture-based and sequence-based methods to characterize the microbiota of germinating seeds and plant cheeses, and to verify the competitiveness of starter cultures. In all samples, Nanopore sequencing of full length 16S rRNA genes (rDNA) confirmed that 98% or more of the rDNA amplicons are derived from one of the strains used as starter culture.

However, the relative abundance of rDNA grossly misrepresented the relative abundance of viable cells, as was previously observed in spontaneous fermentations of plant foods (Pswarayi and Gänzle, 2019; Van der Meulen et al., 2007; Wuyts et al., 2018).

Seeds harbor plant microbiota including plant-associated *Enterobacteriaceae* and bacilli but may also be contaminated with foodborne pathogens including *Salmonella* and pathogenic strains of *E. coli* (Dechet et al., 2014). Treatments for pathogen control include high hydrostatic pressure (Ariefdjohan et al., 2006), high-pressure carbon dioxide (Fang et al., 2021), and chlorine treatment (Montville and Schaffner, 2004). The use of 200 – 20,000 ppm of chlorine reduced cell counts of pathogens on the surface of seeds by 1 – 3 log (cfu/g) without damage to the plant embryo (Montville and Schaffner, 2004; U.S. Food and Drug Administration, 1998). In this study, 2,000 ppm of chlorine treatment inhibited the growth of gamma-proteobacteria and *Bacillota* during seed germination. Colony PCR confirmed that *Pseudomonas* spp. and *Bacillus* spp. were most abundant during seed germination (data not shown). *Pseudomonas* spp. are frequently associated with food spoilage (Møretrø and Langsrud, 2017). However, chlorine solution did not decrease cell counts of *Lactobacillales* present in raw seeds. Protective cultures including *Lp. plantarum* have also been used to control hygienic risks during germination (Budryn et al., 2019; Świeca et al., 2018). In the traditional tempeh fermentation, soaking water acidified by lactic acid bacteria was used to prevent the development of undesirable microbes (Yoneya, 2004). This study confirmed that addition of *Lp. plantarum* and *Lc. lactis* prevented growth of other plant microbiota during seed germination. Thus, chlorine and/or lactic acid bacteria effectively decreased hygienic risks. The fate of pathogens including *Salmonella* during seed germination and production of plant cheeses, however, remains to be determined (Schmitt et al., 2018).

*Lp. plantarum* occurs in many plant food fermentations (Gänzle, 2022; Martino et al., 2016) and served as a starter culture to acidify plant milk (Angelov et al., 2006). Of the selected LAB, *Lp. plantarum* acidified soymilk fastest and was thus selectd as the major starter culture for plant cheese fermentation. Comparable to cheese, *Lc. lactis* also acted as a starter culture for milk acidification. *Lc. lactis* is widely used in mesophilic starter culture for cheese production but also occurs in association with plants (Strafella et al., 2021). This study demonstrated that *Lp. plantarum* and *Lc. lactis* were the dominant microbes in unripened soy and lupine cheeses. *Lc. lactis* died soon during ripening of plant cheese made from ungerminated seeds, matching the inactivation of *Lc. lactis* during cheese ripening (Parente et al., 2017). The presence of bacilli improved the survival of *Lc. lactis* during the ripening of soy or lupine cheeses. LAB generally do not produce extracellular glycosyl hydrolases to degrade non-starch polysaccharides (Gänzle and Follador, 2012; Zheng et al., 2015a) but *Bacillus* spp. produce hydrolytic enzymes (Li et al., 2023b). The presence of bacilli resulted in lower concentrations of sugars in soy and lupine milk, and in a reduced formation of lactic and acetic acids during acidification. The corresponding increased pH of plant cheeses (Table S5.1) apparently supported the survival of *Lc. lactis*. We selected *B. velezensis* FUA2155 and *B. amyloliquefaciens* Fad 82 as representative strains of two of three species of bacilli in the FUA strain collection for which also prior data on their use as beneficial fermentation organisms is available (Li et al., 2023a, 2019; Z. Li et al., 2020). In addition, past studies inform on the control of these strains by lactobacilli (Li et al., 2019). The use of only two strains provides proof of concept on their beneficial contribution to fermentation of plant cheeses but does not represent the metabolic diversity of bacilli with potential for use in food fermentations (Gänzle et al., 2023a; Li et al., 2023b). A more detailed description of their

contribution through quantification of organic acids, amino acids and taste-active peptides, and through sensory analyses remains subject to future investigations.

The microbiota succession in the fermentation of plant cheese was similar to bacteria-type pulse fermentations (Han et al., 2001b), but differed from microbiota observed in cheese ripening. In plant cheese fermentations, the hydrolytic bacilli grew first, followed by the growth of lactic acid bacteria, which inhibited further growth of bacilli by acidification. However, the microbiota sequence is reversed in cheese ripening where lactic starter cultures acidify first, followed by the growth of ripening microorganisms, including non-starter LAB, aerobic bacteria e.g. *Brevibacterium* spp. and *Corynebacterium* spp., mycelial molds, or yeast. Ripening cultures produce proteases and peptidases to develop the flavor and texture of ripened cheese (Cotter and Beresford, 2017). *Lp. plantarum* grows during cheese ripening as an adjunct culture (Cotter and Beresford, 2017), however, this study documented inactivation of *Lp. plantarum* during ripening of plant cheeses, which necessitates the selection of alternative adjunct cultures that retain metabolic activity and maintain microbiome control during extended ripening times.

The release of taste-active amino acids and peptides by proteolysis is a major contributor to the flavor formation of fermented soy foods or ripened cheeses (McSweeney, 2017; Xie et al., 2018). This study revealed that seed germination did not enhance proteolysis of soy or lupine cheeses. *Lp. plantarum* generally lacks genes encoding for extracellular proteinases (Zheng et al., 2015a), however, strains of *Lc. lactis* possess a plasmid-encoded cell-envelope proteinase (PrtP) that degrades casein during cheese ripening (Savijoki et al., 2006). In addition, intracellular peptidases are released after cell death (Lortal and Chapot-Chartier, 2005; McSweeney, 2017). Proteolysis was enhanced by bacilli, indicating that proteases produced by *Bacillus* spp.(Lan et al., 2020) during seed germination remained active during ripening.

Carbohydrate metabolism contributes to flavor formation of fermented foods. Both soy beans and lupine seeds contain sugars and raffinose-family oligosaccharides as well as starch and non-starch polysaccharides (Fritsch et al., 2015; Medic et al., 2014), which can be degraded by glycosyl hydrolases of germinated seeds such as α-galactosidases and α-amylase (Guimarães et al., 2001; Kumari et al., 2010). During acidification of soy and lupine milk, glucose, fructose and sucrose were depleted first while galactose accumulated in some samples, likely owing to hydrolysis of raffinose-family oligosaccharides. Oligosaccharides partially carried over to the cheese curd but were rapidly depleted during ripening, similar to the depletion of residual lactose in ripened cheeses (McSweeney et al., 2017; Portnoi and Macdonald, 2009). However, unknown oligosaccharides were released during extended ripening, influenced by seed germination and/or bacilli, but these compounds remain to be identified.

Malting process as a traditional method for generation of hydrolytic enzymes is frequently used in European cereal fermentations (Geißinger et al., 2022). In contrast, food fermentations in East Asia rely on hydrolytic enzymes produced by mycelial molds or bacilli (Li et al., 2023b). Our study demonstrated that both sources of hydrolytic enzymes can be used for production of plant cheeses, combining the best of both worlds. Hydrolytic enzymes from germinated seeds and bacilli also degrade antinutritive compounds in pulses including phytate, and increases the bioactivity of soy isoflavones (C. Li et al., 2021; Tsuji et al., 2015). In contrast to unfermented and formulated plant cheese products, fermented and ripened plant cheeses can be produced with a minimal number of ingredients or additives and thus registers as minimally processed plant food which not only contains live dietary microbes but also bioactive phytochemicals and dietary fibre and thus likely contribute to a healthy diet (Armet et al., 2022; Hill et al., 2023).

# Chapter 6. Selection of cultures for the ripening of plant cheese analogues

## 6.1 Introduction

The growing evidence that a diet with high levels of plant-based foods improves health and conforms environmental sustainability stimulates the production of healthy and sustainable foods, including plant-based analogues of meat, fish, egg, milk, and cheese products (McClements and Grossmann, 2022). Of these, plant-based cheese analogues, which are subsequently referred to as plant cheese, are normally designed to mimic the sensory attributes of cheese. Plant cheese is either produced from highly refined plant ingredients such as proteins, oils/fats, and starches, etc., or made from whole plant materials such as legumes, grains, and nuts. The use of highly refined plant ingredients causes adverse impacts due to high amounts of energy consumption and addition of saturated plant oil/fats(Lie et al., 2021; McClements and Grossmann, 2022). Plant cheese from whole plant materials that are minimally processed thus exhibits more benefits of environment and human health when compared to these from refined plant ingredients. Challenges in the production of plant cheese from whole plant materials, however, remain to achieve desirable sensory attributes (Grossmann and McClements, 2021).

The fermentation cultures have been used to formulate plant materials to improve sensory profiles of plant cheese, including mono-cultures from dairy cheese and probiotics or mixed-cultures from water kefir, yogurt, cheese, and "rejuvelac" (Chen et al., 2020; Pua et al., 2022). The studies on the selection of functional starter cultures for the fermentation of plant cheese, however, remain limited. The cultures used for the production of plant cheese frequently occur in cheese production such as *Streptococcus thermophilus*, *Lactococcus lactis*, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Lacticaseibacillus spp*, *Lactiplantibacillus plantarum*, and *Geotrichum candidum* (Ben-Harb et al., 2020; Y. Li et al., 2020; Masia et al., 2022), but these isolates from

dairy environment display a limited capacity to ferment plant materials (Kleerebezem et al., 2020; van de Guchte et al., 2006). The microbiota occurring in association with plants or in spontaneous plant fermentation are likely to perform well in plant cheese fermentation (Gänzle, 2022).

The microbiology of traditionally fermented soy foods provides scientific guidance for the control of hygienic risks and the selection of functional starter cultures in plant cheese fermentation (Chapter 2). Chlorine treatment or addition of *Lp. plantarum* and *Lc. lactis* decreased the hygienic risks when plant cheese was made from seeds (Chapter 5). The use of hydrolytic culture *Bacillus* spp. during seed soaking and germination produced extracellular enzymes and enhanced the proteolysis of plant cheeses (Chapter 5), supporting for the growth of non-hydrolytic secondary microbiota. However, Chapter 5 documented the inactivation of LAB during ripening of plant cheeses. It thus necessitates the selection of alternative adjunct cultures that retain metabolic activity during extended ripening times.

Therefore, this study aimed to select functional adjunct cultures for plant cheese ripening and investigate their contributions on the formation of taste-active metabolites, particularly kokumi γ-glutamyl dipeptides, in fermentation of plant cheese. Soy beans or lupine seeds used were germinated with LAB as protector or germinated with LAB and *Bacillus* spp.. Plant-associated lactobacilli that harbor different copies of *gcl* genes encoding for Gcls were selected for ripening microbiota. The plant cheese was ripened for 90 days.

## 6.2 Materials and methods

### 6.2.1 Strains and growth conditions

Starter cultures *Lantiplantibacillus*. *plantarum* TMW1.460 (WEZR01000000) and *Lactococcus lactis* FUA3228 (JAUAPV000000000) were grown in modified deMan-Rogosa-Sharpe (mMRS) medium and M17 medium (Sigma-Aldrich, St. Louis, MO, U.S.A.), respectively,

under microaerophilic conditions at 30 °C. Adjunct cultures that include *Levilactobacillus hammesii* LP38$^T$ (AZFS00000000), *Furfurilactobacillus milli* FUA3115 (JAIWJH000000000), and *Lentilactobacillus buchneri* FUA3252 (JAUAPU000000000) were grown in mMRS medium under microaerophilic conditions at 30 °C. *Bacillus velezensis* FUA2155 (SDKI00000000) and *Bacillus amyloliquefaciens* Fad 82 (SDKG00000000) were grown in Luria-Bertani (LB) medium (BD, Mississauga, Ontario, Canada) under aerobic conditions at 37 °C. Cells harvested from overnight cultures were washed twice with equal volumes of 0.85% saline prior to fermentation.

### 6.2.2 Materials and chemicals

Soy beans were obtained in a local supermarket; lupine seeds were kindly provided by Lumi Foods (Vancouver, Canada). Standards of γ-glutamyl dipeptides (γ-Glu-Cys, γ-Glu-Ala, γ-Glu-Val, γ-Glu-Ile, γ-Glu-Leu, γ-Glu-Phe, γ-Glu-Met, γ-Glu-Pro, γ-Glu-Gly, γ-Glu-Ser, γ-Glu-Glu, γ-Glu-Gln and γ-Glu-Asp) were purchased from United Biosystems (Herndon, VA, U.S.A.). Standards of L-lithium lactate, sodium acetate, 1,2-propanediol, propionic acid, galactose, glucose, fructose, raffinose, stachyose, and verbascose were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Dithiothreitol (DTT), iodoacetamide (IAM), 5-sulfosalicylic acid (SSA), ammonium hydroxide solution, acetonitrile, and methanol were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

### 6.2.3 Preparation and ripening of soy and lupine cheeses

Soy beans or lupine seeds were soaked with 0.2% chlorine solution for 30 min, rinsed twice using sterile $H_2O$, and soaked with the cell suspension of *Lp. plantarum* and *Lc. lactis* with or without *B. velezensis* or *B. amyloliquefaciens* for 2 h. The cell count of each inoculum in soaking cultures ranged from $10^7$ to $10^8$ cfu/ml. The cell suspensions were drained after soaking. Soaked soy beans or lupine seeds were subsequently germinated on sterile plates in a sterile container with

100% relative humidity (rH) at 30 °C for 2 d. Germinated seeds were washed twice and blended into the plant milk after addition of sterile $H_2O$ in a ratio of 1:5 (w/v). The ungerminated plant milk samples were made from ungerminated soy beans or lupine seeds that were pre-treated with 0.2% chlorine solution for 30 min. The adjunct *Lv. hammesii*, *Ff. milli*, or *Lt. buchneri* was inoculated into the plant milk sample with an initial cell count of around $10^7$ cfu/ml.

Soy or lupine milk was incubated at 30 °C for 12 h and proteins were precipitated. Soy or lupine cheeses were obtained by centrifugation of acidified plant milk at 3500 x g for 10 min. Cheese samples were subsequently incubated at 8 °C and rH 0.88 for the ripening of 0, 14, 45, and 90 d. Cell counts were determined with fresh cheese samples using LB, mMRS, and M17 agar plates. The colony morphology of all colonies on the agar plates was compared to the colony morphology of the inocula. The 16S rRNA gene amplicons from community DNA were sequenced to verify conclusions based on the morphology of colonies on agar plates. The freezer-dried cheese was stored at – 20 °C until use for the analysis of carbohydrates, glutamate, lactate, acetate, 1,2-propanediol, propionic acid, γ-glutamyl dipeptides, and sequencing of bacteria. The ripening of soy or lupine cheese was performed with three biological replicates. Results are reported as means ± standard deviation.

### 6.2.4 Quantitation of γ-glutamyl dipeptides by LC-MS/MS in cheese samples

The methods to quantify γ-glutamyl dipeptides using LC-MS/MS were similar as described (Lin et al., 2023). LC-MS/MS parameters for quantification of 13 kokumi peptides are shown in Table 6.1.

**Table 6.1.** LC−MS/MS parameters for quantitation of γ-glutamyl dipeptides.

| Analyte | Ion transition (m/z) | Retention time (min) | Cone voltage (V) | Collision energy (V) |
|---|---|---|---|---|
| InfinityLab Poroshell 120 HILIC-Z, P column | | | | |
| γ-Glu-Cys | [a)]308.2/179.1 | 7.75 | 15 | 19 |
| γ- Glu-Gly | 205.1/76.05 | 7.90 | 15 | 13 |
| γ- Glu-Pro | 245.2/116.12 | 6.66 | 15 | 15 |
| γ- Glu-Ala | 219.2/90.08 | 6.88 | 15 | 14 |
| γ- Glu-Ser | 235.2/106.08 | 7.82 | 14 | 15 |
| γ-Glu-Gln | 276.2/147.1 | 7.92 | 14 | 16 |
| γ- Glu-Asp | 263.2/134.1 | 7.94 | 15 | 15 |
| γ-Glu-Glu | 277.2/148.1 | 7.76 | 15 | 16 |
| γ-Glu-Met | 279.2/150.2 | 5.88 | 15 | 16 |
| γ-Glu-Val | 247.2/118.1 | 5.90 | 15 | 15 |
| Luna Omega polar C18 column | | | | |
| γ-Glu-Phe | 295.3/166.2 | 4.70 | 15 | 18 |
| γ-Glu-Leu | 261.2/132.1 | 3.90 | 15 | 15 |
| γ-Glu-Ile | 261.2/132.1 | 3.68 | 15 | 15 |

[a)] γ-Glu-Cys alkylated with IAM was used in LC-MS/MS analysis.

γ-Glutamyl dipeptides were extracted from ~ 0.1 g of dry cheese sample using 1 ml of Milli-Q water by the incubation at 25 °C with agitation 250 rpm for 90 min, followed by centrifugation at 17000 x g for 5 min. Three hundred µl of supernatants were adjusted to obtain a final pH of about 8.5 using ammonium hydroxide solution, mixed with 30 µl of 100 mM DTT (pH >8.5), and incubated for 30 min at 50 °C to reduce disulfide linkage. Subsequently, 30 µl of 200 mM IAM was added into the mixture, followed by incubation in dark at ambient temperature for 20 min to alkylate γ-Glu-Cys. The resulting mixture was finally acidified to pH of 3.0 – 3.5 by 15 µl of 500 mM SSA. Alkylated sample was dried using Savant SpeedVac vacuum concentrators (Thermo Scientific), resuspended in 200 µl of 100% methanol with 0.1% formic acid (FA, v/v),

and centrifuged at 13000 x g for 5 min to remove precipitates. The supernatant was transferred into clean tube and dried for additional 20 min using Savant SpeedVac vacuum concentrators to remove methanol. Finally, the resulting γ-glutamyl dipeptides were resuspended in 200 µl of 45% acetonitrile with 0.1% FA for LC-MS/MS analysis. γ-Glu-Cys standard was derivatized as described above. Standard mixtures of 10 γ-glutamyl dipeptides (γ-Glu-Ala, γ-Glu-Val, γ-Glu-Gly, γ-Glu-Ser, γ-Glu-Met, alkylated γ-Glu-Cys, γ-Glu-Pro, γ-Glu-Gln, γ-Glu-Glu, and γ-Glu-Asp) with different concentrations ranging from 1.0 to 20.0 ng/µl were prepared using the solution of 45% acetonitrile with 0.1% FA.

For the extraction and quantification of γ-Glu-Ile, γ-Glu-Leu, and γ-Glu-Phe, samples were not derivatized using IAM. Three hundred µl of the aqueous extract was dried using Savant SpeedVac vacuum concentrators (Thermo Scientific), resuspended in 200 µl of 100% methanol with 0.1% formic acid (FA, v/v), and centrifuged at 13000 x g for 5 min to remove precipitates. The supernatant was transferred into clean tube and dried for additional 20 min using Savant SpeedVac vacuum concentrators to remove methanol. Finally, the resulting γ-glutamyl dipeptides were resuspended in 200 µl of Milli-Q water with 0.1% FA for LC-MS/MS analysis. The standard mixtures of γ-Glu-Ile, γ-Glu-Leu, and γ-Glu-Phe with different concentrations ranging from 0.01 to 10.0 ng/µl were prepared using Milli-Q $H_2O$ with 0.1% FA.

LC-MS/MS analyses were completed by service of the Mass Spectrometry service facility of the Dept. of Chemistry, University of Alberta. γ-Glutamyl dipeptides, including γ-Glu-Ala, γ-Glu-Val, γ-Glu-Gly, γ-Glu-Ser, γ-Glu-Met, alkylated γ-Glu-Cys, γ-Glu-Pro, γ-Glu-Gln, γ-Glu-Glu, and γ-Glu-Asp, were separated using an InfinityLab Poroshell 120 HILIC-Z, P column (2.7 µm, 50 × 2.1 mm, Phenomenex, Torrance, CA, U.S.A.) in an Agilent 1100 series HPLC unit and detected using a Micromass Quattro micro API tandem quadrupole LC-MS/MS system (Waters

Corporation, Milford, Massachusetts, U.S.A) with multiple reaction monitoring (MRM) mode. Mobile phase A and B consisted of Milli-Q water with 0.1 % FA and 0.1 % ammonium formate ($NH_4FA$) and 90 % acetonitrile with 0.1 % FA and 0.1 % $NH_4FA$, respectively. Samples were eluted at a flow of 0.6 ml/min as the following gradients: 0−0.50 min, 100% B; 0.50−11.00 min, 100−70% B; 11.00−12.00 min, 70−100% B; and followed by a column re-equilibration with 100% B for 6 min at a flow of 0.6 ml/min. Data acquisition was interfaced to the Masslynx v4.1 software (Waters Corporation, Milford, Massachusetts, U.S.A).

γ-Glu-Ile, γ-Glu-Leu, and γ-Glu-Phe were separated using a Wasters Acquity UPLC unit equipped with a Luna Omega polar C18 column (1.6 µm, 50 × 2.1 mm, Phenomenex, Torrance, CA, U.S.A.) and detected using a Xevo TQ MS system (Waters Corporation, Milford, Massachusetts, U.S.A) with multiple reaction monitoring (MRM) mode. Mobile phase A and B consisted of Milli-Q water with 0.1 % FA and acetonitrile with 0.1 % FA, respectively. Samples were eluted as the following flow rates and gradients: 0−0.50 min, 0.3 ml/min, 99% A; 0.50−6.00 min, 0.3 ml/min, 99−88% A; 6.00−8.00 min, 0.3 ml/min, 88−80% A; 8.00−8.01, 0.3 ml/min, 80−5% A; 8.01−10.00, 0.5 ml/min, 5% A; 10.00−10.10, 0.5 ml/min, 5−99% A; and followed by a column re-equilibration with 99% A for 5 min at a flow of 0.5 ml/min. Data acquisition was interfaced to the Masslynx v4.1 software (Waters Corporation, Milford, Massachusetts, U.S.A).

### 6.2.5 Quantification of carbohydrates in soy and lupine cheeses

Carbohydrates including mono-, di-, and oligosaccharides were extracted from ~ 0.05 g of dry soy or lupine cheese using 1 ml of sodium phosphate buffer (100 mmol $L^{-1}$, pH 7.4) by the shaking incubation at 250 rpm and 25 °C for 1 h and by centrifugation at 17,000 x g for 10 min. The aqueous extract was diluted 10-fold and stored at – 20 °C prior to analysis. The quantification of mono-, di-, and oligosaccharides was performed using HPAEC-PAD with a Carbopac PA20

column coupled to an ED40 chemical detector on a Dionex ICS 6000 unit (Dionex, Oakville, Canada). Mobile phase A, B, and C consist of Milli-Q $H_2O$, 0.2 M sodium hydroxide, and 1 M sodium acetate, respectively. Samples were eluted at a flow of 0.25 ml $min^{-1}$ at the following gradients: 0 – 30 min, 15% B, 0 – 1.8% C; 30 – 40 min, 15 – 0% B, 1.8 – 50% C; 40 – 45 min, 0 – 40% B, 50 – 0% C; 45 – 55 min, 40 – 80% B, 0% C; 55 – 60 min, 80 – 15% B, 0% C; 60 – 80 min, 15% B, 0% C. Standards of galactose, glucose, fructose, sucrose, melibiose, raffinose, stachyose, and verbascose served as external standards, ranging from 0.1 to 10.0 mg $L^{-1}$

### 6.2.6 Determination of free amino nitrogen and glutamate

To determine the concentrations of free amino nitrogen in plant cheeses, the sample preparation was the same as described in carbohydrate quantification above. The aqueous extract was diluted 100-fold using distilled water. Two hundred µl of diluted extract was mixed with 100 µl of ninhydrin solution, incubated at 100 °C for 16 min, cooled to room temperature for 20 min, and mixed with 500 µl of $KIO_3$ solution. The absorbance was determined at 570 nm using a microplate reader (Varioskan Flash, Thermo Electron Corporation, CA, USA). Glycine served as an external standard to create a calibration curve.

To assay glutamate in soy cheese, ~ 0.1 g of dry soy cheese was homogenized with 1 ml of Milli-Q water at 25 °C and 250 rpm for 90 min and centrifuged at 17,000 x g for 10 min to obtain the aqueous extract. The glutamate concentration in the aqueous extract was determined using a Glutamate Assay Kit according to the instructions of manufacturer (Sigma-Aldrich, St. Louis, MO, U.S.A.). Twenty-five µl of sample was mixed well with 50 µl of background control mix without addition of enzyme mix or 50 µl of reaction mix with addition of enzyme mix by pipetting and incubated at 37 °C for 30 min with the protection from light. The absorbance was

measured at 450 nm using a microplate reader. One mm $L^{-1}$ of glutamate solution was used to make a calibration curve of external standards.

### 6.2.7 Quantification of organic acids and 1,2-propanediol by HPLC

For quantification of organic acids and 1,2-propanediol, the aqueous extract was prepared by homogenization of ~ 0.2 g of dry soy cheese with 0.9 ml of Milli-Q water at 25 °C and 250 rpm for 90 min and centrifuged at 17,000 x g for 10 min. The aqueous extract was mixed with 7% perchloric acid solution in a ration of 1:1 (v/v) and incubated at room temperature for overnight, following by centrifugation at 17,000 x g for 10 min to remove precipitates. The resulting supernatant was transferred into a clean tube and stored at – 20 °C until use for quantification. Lactate, acetate, propionic acid, and 1,2-propanediol were separated using an Aminex HPX-87H column in an Agilent 1200 series HPLC system and detected under the refractive index detector and UV detector. Samples were eluted at 70 °C and a flow rate of 0.4 ml $L^{-1}$ with 5 mM $H_2SO_4$. The calibration curves of external standards were generated by L-lithium lactate, sodium acetate, propionic acid, and 1,2-propanediol with concentrations from 1 to 25 mM $L^{-1}$.

### 6.2.8 DNA isolation and full length 16S rRNA gene sequencing

To verify the identity of plant cheese microbiota with strain inocula, the sequencing of full length 16S rRNA gene amplicons was performed by nanopore machine MinION. Genomic DNA of cheese microbiota was isolated using a DNeasy Blood and Tissue Kit (Qiagen, Mississauga, ON, Canada). For sequencing, DNA libraries were prepared using 16S Barcoding Kit 1-24 (SQK-16S024) and Flongle Sequencing Expansion Kit (EXP-FSE001), then loaded into the R9.4.1 Flongle flow cell following the instructions of manufacturer (Oxford Nanopore Technologies, Oxford Science Park, Oxford, United Kingdom). Data acquisition and high accuracy basecalling were performed with MinKNOW v22.12.7 and Guppy v6.4.6, respectively; the basecalled data

was assayed in EPI2ME Labs wf-metagenomics v2.0.8. software using NCBI 16S and 18S rRNA database.

### 6.2.9 Statistical analysis

Data analysis for concentrations of carbohydrates, organic acids, γ-glutamyl dipeptides, free amino nitrogen, and glutamate in soy and lupine cheese samples was performed by one-way analysis of variance (ANOVA) using IBM SPSS statistics 23. *P* value of ≤0.05 with Tukey adjustment for multiple comparisons was considered statistically significant.

## 6.3 Results

### 6.3.1 Microbial cell counts during plant cheese ripening

The cell counts of starter cultures during cheese ripening were monitored by a culture-dependent method. The colony morphology of all colonies from all samples on agar plates was consistent with the colony morphology of one of the four strains used as stater cultures. The sequence of more than 98% of 16S rRNA gene amplicons from community DNA of cheese samples matched the sequences of 16S rRNA genes of strains used as starter cultures (Table S6.1 and Table S6.2), indicating that these cultures dominated the microbiota of plant cheeses and controlled growth of other microbes.

The cell counts of *B. velezensis* in unripened soy and lupine cheeses were 8 – 9 log (cfu/g) and 7 – 8 log (cfu/g) (Figure 6.1 and Figure 6.2), respectively. Cell counts of *B. amyloliquefaciens* were one log lower than those of *B. velezensis* in unripened soy cheeses. Cell counts of bacilli remained stable during the ripening of soy and lupine cheeses, indicating survival of endospores rather than vegetative cells. Cell counts of *Lp. plantarum* and *Lc. lactis* in unripened plant cheeses ranged from 10 – 11 log (cfu/g) and 9 – 10 log (cfu/g) (Figure 6.1 and Figure 6.2), respectively. *Lc. lactis* died and their cell counts decreased to levels below the detection limit during cheese

ripening, but the addition of bacilli during seed germination improved the survival of *Lc. lactis* during cheese ripening (Figure 6.1 and Figure 6.2). After the ripening of 90 days, cell counts of *Lp. plantarum* decreased by 2 – 3 log (cfu/g) in plant cheeses. However, this reduction was irrespective of germination, bacilli, or adjuncts. Cell counts of *Lv. hammesii*, *Fr. milli*, and *Lt. buchneri* in unripened plant cheeses were 8 – 9 log, 9 – 10 log, and 8 – 9 log (cfu/g) (Figure 6.1 and Figure 6.2), respectively. Cell counts of adjunct cultures remained stable throughout 45 d of ripening; *Ff. milli* and *Lt. buchneri* grew slowly during plant cheese ripening.

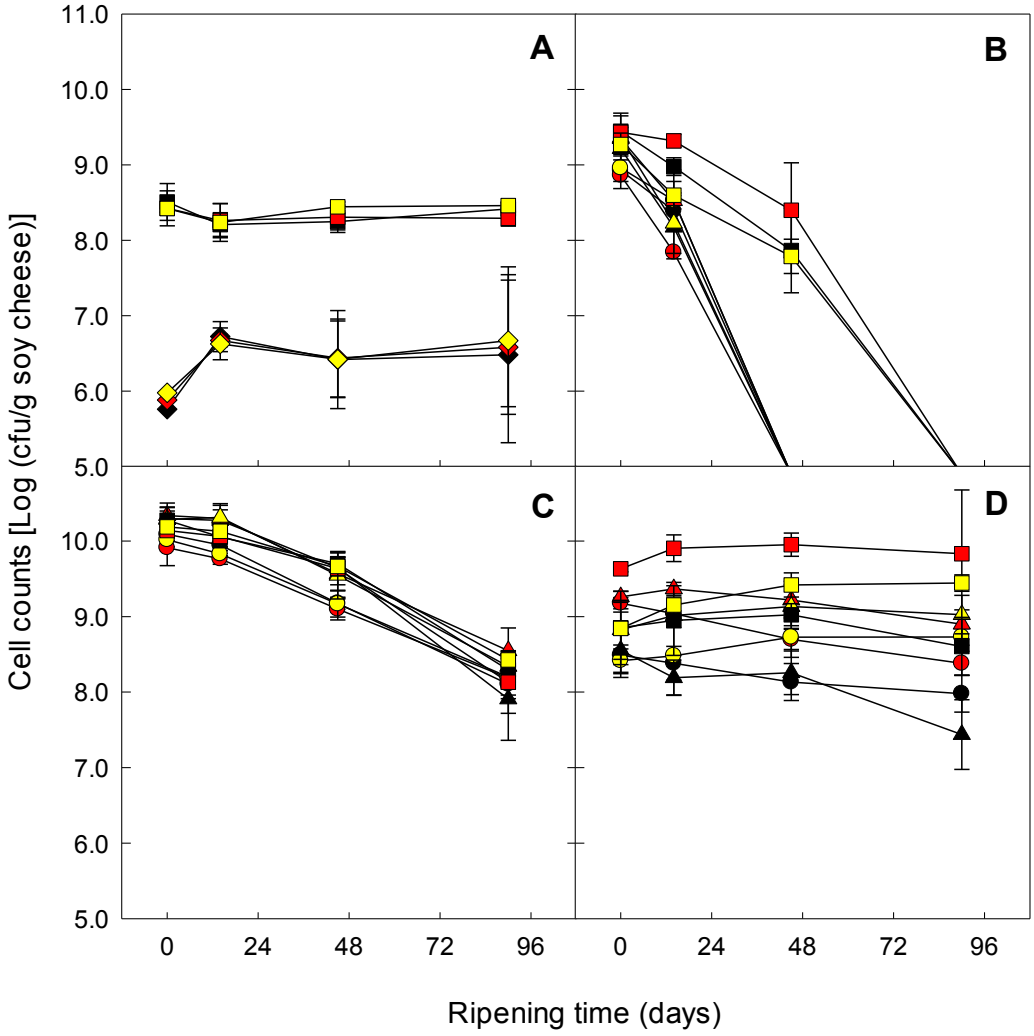



**Figure 6.1.** Cell counts of A) *Bacillus* spp., B) *Lc. lactis*, C) *Lp. plantarum*, and D) Lactobacilli inoculates in soy cheeses ripened for 0, 14, 45, and 90 days. Symbols indicate cell counts for soy cheeses from ungerminated seeds (circle), or seeds germinated: with LAB only (triangle), or LAB with *B. velezensis* (square) or with *B. amyloliquefaciens* (diamond). Colours indicate the cell counts for soy cheeses inoculated with: *Lv. hammesii* (black), *Ff. milii* (red), and *Lt. buchneri* (yellow). The y-axis was scaled to the detection limit of $10^5$ cfu/g and lines dropping below the x-axis indicate values below the detection limit.

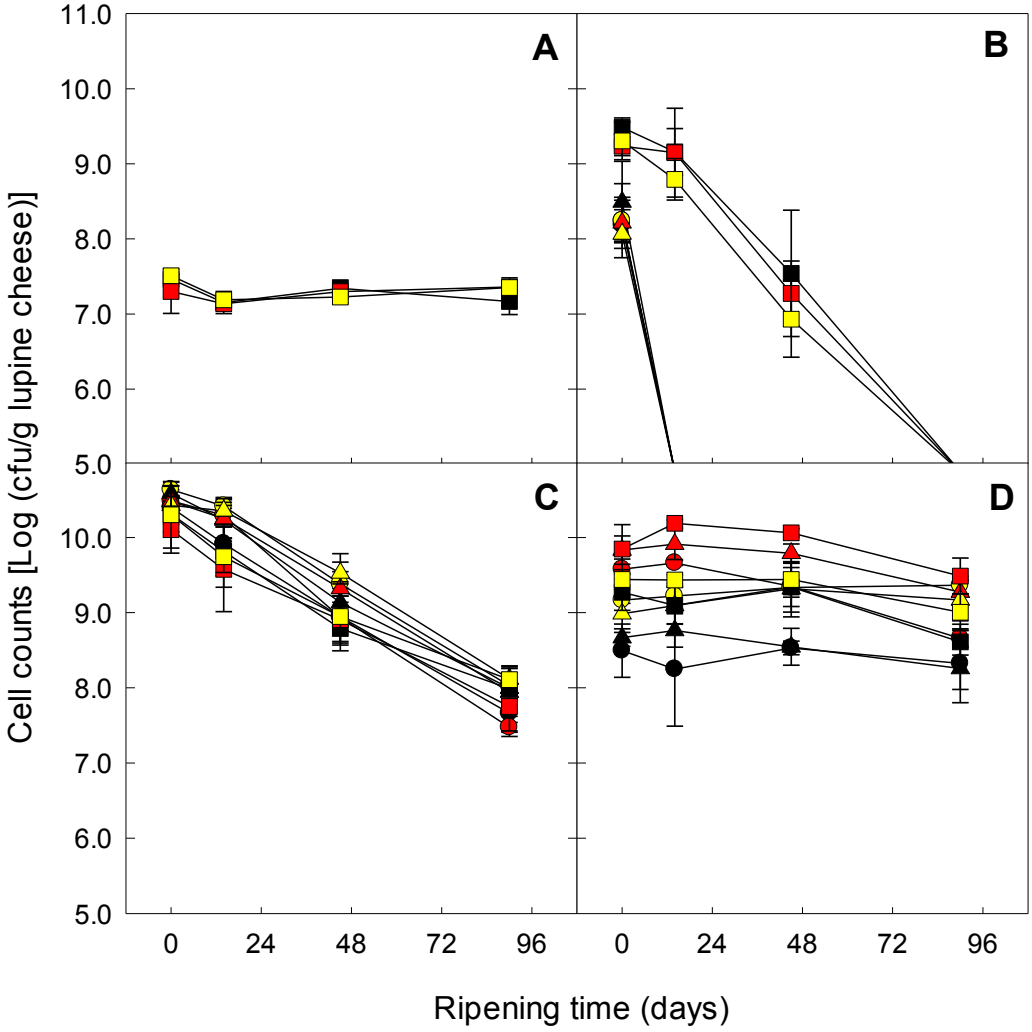



**Figure 6.2.** Cell counts of A) *Bacillus* spp., B) *Lc. lactis*, C) *Lp. plantarum*, and D) Lactobacilli inoculates in lupine cheeses ripened for 0, 14, 45, and 90 days. Symbols indicate the cell counts for lupine cheeses from ungerminated seeds (circle), or seeds germinated: with LAB only (triangle), or LAB with *B. velezensis* (square) or with *B. amyloliquefaciens* (diamond). Colours indicate the cell counts for lupine cheeses inoculated with: *Lv. hammesii* (black), *Ff. milii* (red), and *Lt. buchneri* (yellow). The y-axis was scaled to the detection limit of $10^5$ cfu/g and lines dropping below the x-axis indicate values below the detection limit.

### 6.3.2 Contribution of seed germination and adjunct cultures to the accumulation of organic acids and 1,2-propanediol

Sugars and metabolites of carbohydrate metabolism including lactic acid, acetic acid, propionic acid and 1,2-propanediol were quantified in soy cheeses. In unripened soy and lupine

cheeses, concentrations of galactose, raffinose, stachyose, and verbascose of ungerminated samples were higher than those in germinated samples (Figure S6.1 and Figure S6.2). Mono-, and oligosaccharides were depleted after 14 d of ripening (data not shown). The concentration of lactic acid was higher in soy cheese from ungerminated seeds than in soy cheese from germinated seeds, but the concentration of acetic acid was lower (Table 6.2). The differences in the concentrations of lactic acid and acetic acid between cheeses from ungerminated seeds and from germinated seeds might thus relate to their different carbohydrate concentrations. The concentrations of propionic acid in all cheese samples remained unchanged. Concentration of organic acids in samples fermented with *Lv. hammesii* and *Ff. milii* were not different (Table 6.2). In plant cheeses fermented with *Lt. buchneri*, the lactic acid concentration was lower, and the acetic acid concentration was higher when compared to other adjunct lactobacilli. 1,2-Propanediol was detected only in ripened soy cheeses with an adjunct *Lt. buchneri*, indicating that *Lt. buchneri* but not *Lv. hammesii* and *Ff. milii* converted lactic acid to 1,2-propanediol and acetic acid during cheese ripening.

**Table 6.2.** Concentrations of organic acids and 1,2-propanediol in soy cheeses with adjunct cultures ripened for 90 days. Soy cheeses were made from ungerminated seeds or seeds germinated: with LAB only, or LAB with *B. velezensis*.

| Samples | Conc. (µmol/g dry weight) | | |
|---|---|---|---|
| | lactic acid | acetic acid | 1,2-propanediol |
| Ungerminated | | | |
| No adjunct culture | 384.3 ± 62.6 | 74.9 ± 6.5 | [a)]n.d. |
| *Lv. hammesii* | 384.2 ± 13.1 | 78.0 ± 14.2 | [a]n.d. |
| *Ff. milii* | 387.6 ± 100.3 | 54.8 ± 19.5 | [a)]n.d. |
| *Lt. buchneri* | 305.7 ± 66.7 | 103 ± 23.4 | 9.4 ± 6.7 |
| Germinated with LAB | | | |
| No adjunct culture | 58.6 ± 66.4 | 136.3 ± 55.3 | [a)]n.d. |
| *Lv. hammesii* | 49.8 ± 32.8 | 148.8 ± 18.6 | [a)]n.d. |
| *Ff. milii* | 53.3 ± 42.7 | 149.1 ± 28.4 | [a)]n.d. |
| *Lt. buchneri* | 1.9 ± 1.3 | 179.2 ± 7.3 | 1.8 ± 0.2 |
| Germinated with LAB and *B. velezensis* | | | |
| No adjunct culture | 21.4 ± 15.3 | 138.5 ± 11.1 | [a)]n.d. |
| *Lv. hammesii* | 11.6 ± 14.4 | 142.2 ± 4.6 | [a)]n.d. |
| *Ff. milii* | 8.6 ± 4.7 | 152.0 ± 21.4 | [a)]n.d. |
| *Lt. buchneri* | 3.7 ± 4.6 | 148.5 ± 14.6 | 2.4 ± 0.5 |

[a)] not detected.

### 6.3.3 Changes in concentrations of glutamate and free amino nitrogen during plant cheese ripening

Glutamate as a taste-active amino acid was assayed during soy cheese ripening. Concentration of free amino nitrogen as an index of proteolysis was quantified. Germination did not change the concentrations of glutamate or free amino nitrogen. In unripened cheeses made from soy beans that were germinated with LAB and *B. velezensis*, the concentration of glutamate was higher ($P< 0.05$) than in samples from ungerminated soy beans or soy beans germinated with LAB only or with LAB and *B. amyloliquefaciens* (Table 6.3). The use of *B. velezensis* increased the concentration of free amino nitrogen of soy cheeses but not lupine cheeses (Table S6.3 and

Table S6.4). The concentrations of glutamate and free amino nitrogen in soy cheese ripened for 45 days were higher ($P< 0.05$) than in unripened soy cheese, indicating that glutamate and free amino nitrogen accumulated during ripening. The presence of adjunct lactobacilli during ripening did not impact the change of total free amino nitrogen but enhanced the accumulation of glutamate in soy cheese from seeds germinated with LAB and *B. velezensis*.

**Table 6.3.** Concentrations of glutamate in soy cheeses with adjunct cultures ripened for 0, 14, 45, and 90 days. Soy cheeses were made from ungerminated seeds or seeds germinated: with LAB only, or LAB with *B. velezensis* or with *B. amyloliquefaciens*.

| Samples | Conc. (µmol/g dry weight) | | | |
|---|---|---|---|---|
| | 0 d | 14 d | 45 d | 90 d |
| Non-adjunct culture | | | | |
| ungerminated | -0.03±0.03$^{ab}$ | 0.00±0.04$^{a}$ | 0.01±0.02$^{b}$ | 0.04±0.02$^{b}$ |
| Germinated with LAB | -0.14±0.29$^{b}$ | 0.02±0.03$^{a}$ | 0.01±0.01$^{b}$ | 0.02±0.04$^{b}$ |
| Germinated with LAB and *B. velezensis* | 0.38±0.16$^{a}$ | 0.08±0.02$^{a}$ | 0.83±0.53$^{a}$ | 1.64±1.05$^{a}$ |
| Germinated with LAB and *B. amyloliquefaciens* | 0.02±0.01$^{ab}$ | 0.08±0.07$^{a}$ | 0.16±0.07$^{ab}$ | 0.24±0.16$^{b}$ |
| *Lv. hammesii* | | | | |
| ungerminated | 0.09±0.11$^{b}$ | -0.01±0.04$^{a}$ | -0.03±0.05$^{b}$ | 0.00±0.06$^{b}$ |
| Germinated with LAB | 0.01±0.02$^{b}$ | 0.09±0.11$^{a}$ | 0.10±0.07$^{b}$ | 0.05±0.07$^{b}$ |
| Germinated with LAB and *B. velezensis* | 0.30±0.07$^{a}$ | 0.15±0.03$^{a}$ | 1.57±0.34$^{a*}$ | 1.89±1.17$^{a}$ |
| Germinated with LAB and *B. amyloliquefaciens* | 0.01±0.02$^{b}$ | -0.02±0.06$^{a}$ | 0.09±0.12$^{b}$ | 0.33±0.11$^{b}$ |
| *Ff. milii* | | | | |
| ungerminated | -0.15±0.06$^{b}$ | 0.00±0.02$^{b}$ | 0.00±0.02$^{b}$ | 0.02±0.07$^{b}$ |
| Germinated with LAB | 0.02±0.06$^{b}$ | 0.02±0.01$^{b}$ | 0.06±0.10$^{b}$ | 0.05±0.07$^{b}$ |
| Germinated with LAB and *B. velezensis* | 0.66±0.23$^{a}$ | 0.33±0.18$^{a}$ | 1.46±0.38$^{a*}$ | 1.92±1.03$^{a}$ |
| Germinated with LAB and *B. amyloliquefaciens* | 0.05±0.05$^{b}$ | 0.07±0.11$^{ab}$ | 0.20±0.20$^{b}$ | 0.48±0.5$^{ab}$ |
| *Lt. buchneri* | | | | |
| ungerminated | -0.16±0.15$^{b}$ | 0.03±0.03$^{a}$ | -0.03±0.11$^{b}$ | -0.05±0.08$^{b}$ |
| Germinated with LAB | 0.02±0.05$^{b}$ | 0.00±0.01$^{a}$ | -0.02±0.06$^{b}$ | -0.04±0.02$^{b}$ |
| Germinated with LAB and *B. velezensis* | 0.39±0.11$^{a}$ | 0.15±0.01$^{a}$ | 1.20±0.39$^{a*}$ | 1.80±0.97$^{a}$ |
| Germinated with LAB and *B. amyloliquefaciens* | -0.03±0.08$^{b}$ | 0.11±0.11$^{a}$ | 0.11±0.10$^{b}$ | 0.38±0.31$^{b}$ |

Values for each adjunct culture in the same column that do not share a lowercase superscript differ significantly ($P<0.05$). Significant differences ($P<0.05$) between unripened soy cheeses and soy cheeses ripened for 45 days are indicated with an asterisk.

### 6.3.4 Quantification of γ-glutamyl dipeptides in plant cheeses

Thirteen γ-glutamyl dipeptides were quantified in plant cheeses. These γ-glutamyl dipeptides were either reported as kokumi taste-active γ-glutamyl dipeptides or synthesized by γ-glutamyl-cysteine ligases from lactobacilli (Zhao et al., 2016). In soy cheeses from ungerminated seeds fermented with adjunct *Lt. buchneri*, the concentrations of γ-Glu-Val, γ-Glu-Ile and γ-Glu-Leu were higher ($P$<0.05) than in soy cheeses without adjunct or with *Lv. hammesii* or *Ff. milii* (Figure 6.3, Figure 6.4 and Figure 6.5). γ-Glu-Ile and γ-Glu-Leu were accumulated by *Lt. buchneri* but not by *Lv. hammesii* or *Ff. milii* during ripening of soy cheeses from seeds germinated with LAB only or with LAB and *B. amyloliquefaciens* (Figure 6.4 and Figure 6.5). In lupine cheeses, *Lt. buchneri* also accumulated γ-Glu-Ile in during ripening. *Ff. milii* accumulated γ-Glu-Ile only in lupine cheeses from seeds germinated with LAB and *B. velezensis* (Figure 6.6). In contrast, accumulation of γ-Glu-Leu during ripening was not observed in any lupine cheese (Figure 6.7). These results indicated the germination, bacilli, or adjuncts impacted the accumulation of γ-Glu-Val, γ-Glu-Ile, and γ-Glu-Leu during plant cheese ripening.

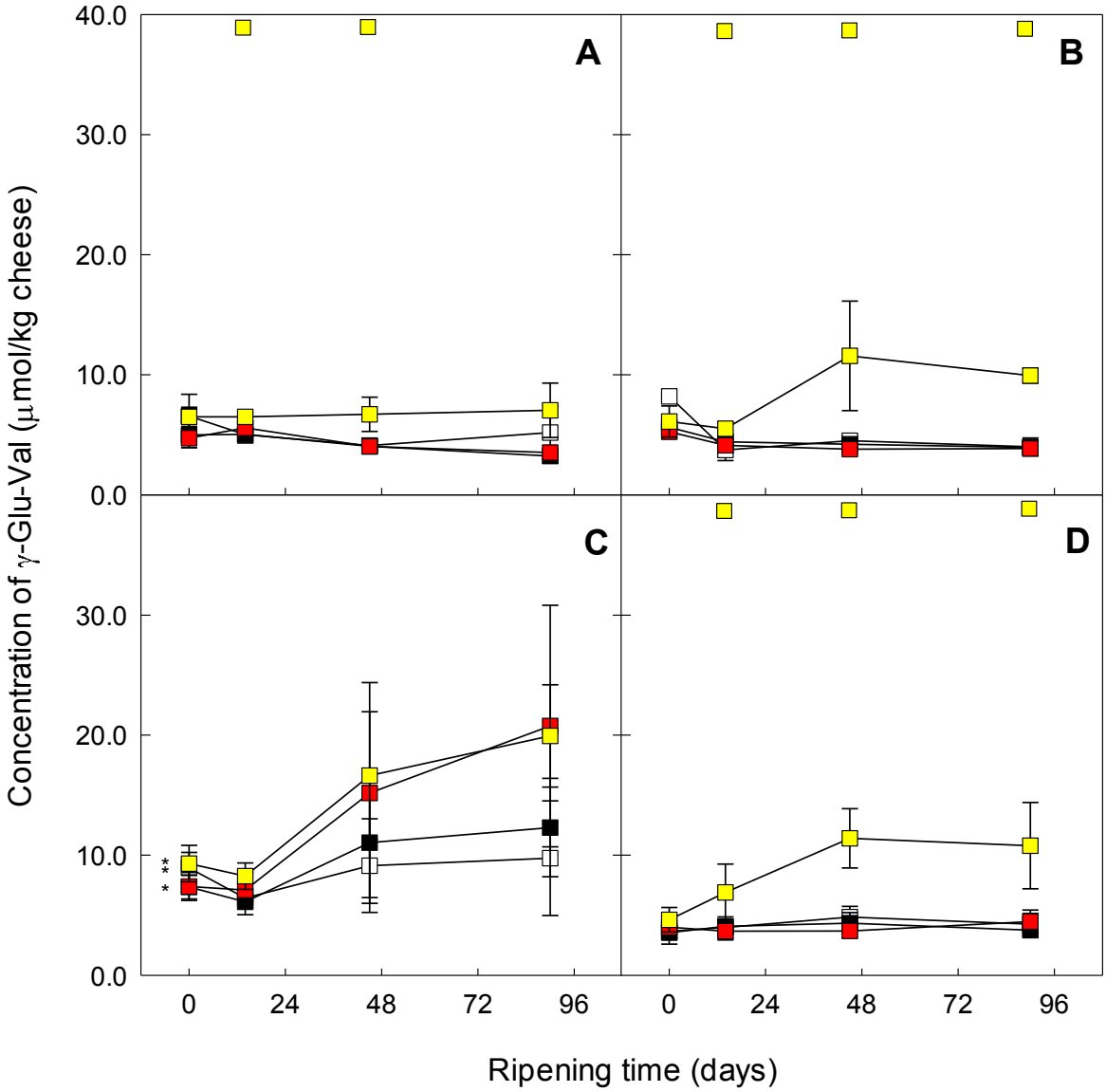



**Figure 6.3.** Concentration of γ-Glu-Val in soy cheeses from ungerminated seeds (A) or seeds germinated: with LAB only (B), or LAB with *B. velezensis* (C) or with *B. amyloliquefaciens* (D). Colours indicate the concentration of γ-Glu-Val in soy cheeses inoculated without adjunct strain (white), or with *Lv. hammesii* (black), *Ff. milii* (red), and *Lt. buchneri* (yellow). The symbols on the top x-axis indicated the corresponding adjuncts were significantly different ($P$<0.05) with non-adjunct control. In unripened soy cheeses, significant differences ($P$<0.05) between cheeses from ungerminated seeds and from germinated seeds were indicated with an asterisk.

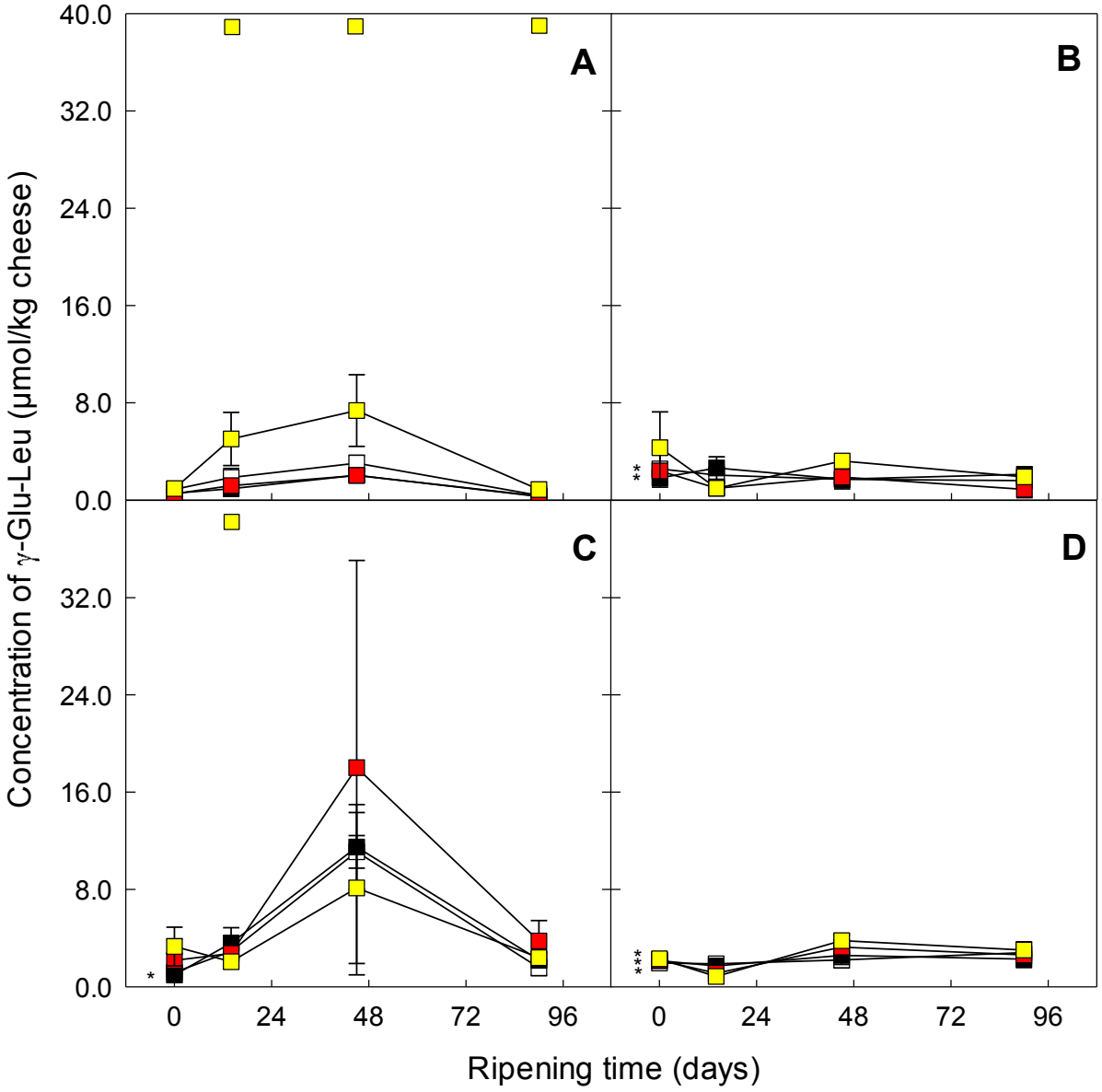



**Figure 6.4.** Concentration of γ-Glu-Leu in soy cheeses from ungerminated seeds (A) or seeds germinated: with LAB only (B), or LAB with *B. velezensis* (C) or with *B. amyloliquefaciens* (D). Colours indicate the concentration of γ-Glu-Leu in soy cheeses inoculated without adjunct strain (white), or with *Lv. hammesii* (black), *Ff. milii* (red), and *Lt. buchneri* (yellow). The symbols on the top x-axis indicated the corresponding adjuncts were significantly different ($P$<0.05) with non-adjunct control. In unripened soy cheeses, significant differences ($P$<0.05) between cheeses from ungerminated seeds and from germinated seeds were indicated with an asterisk.

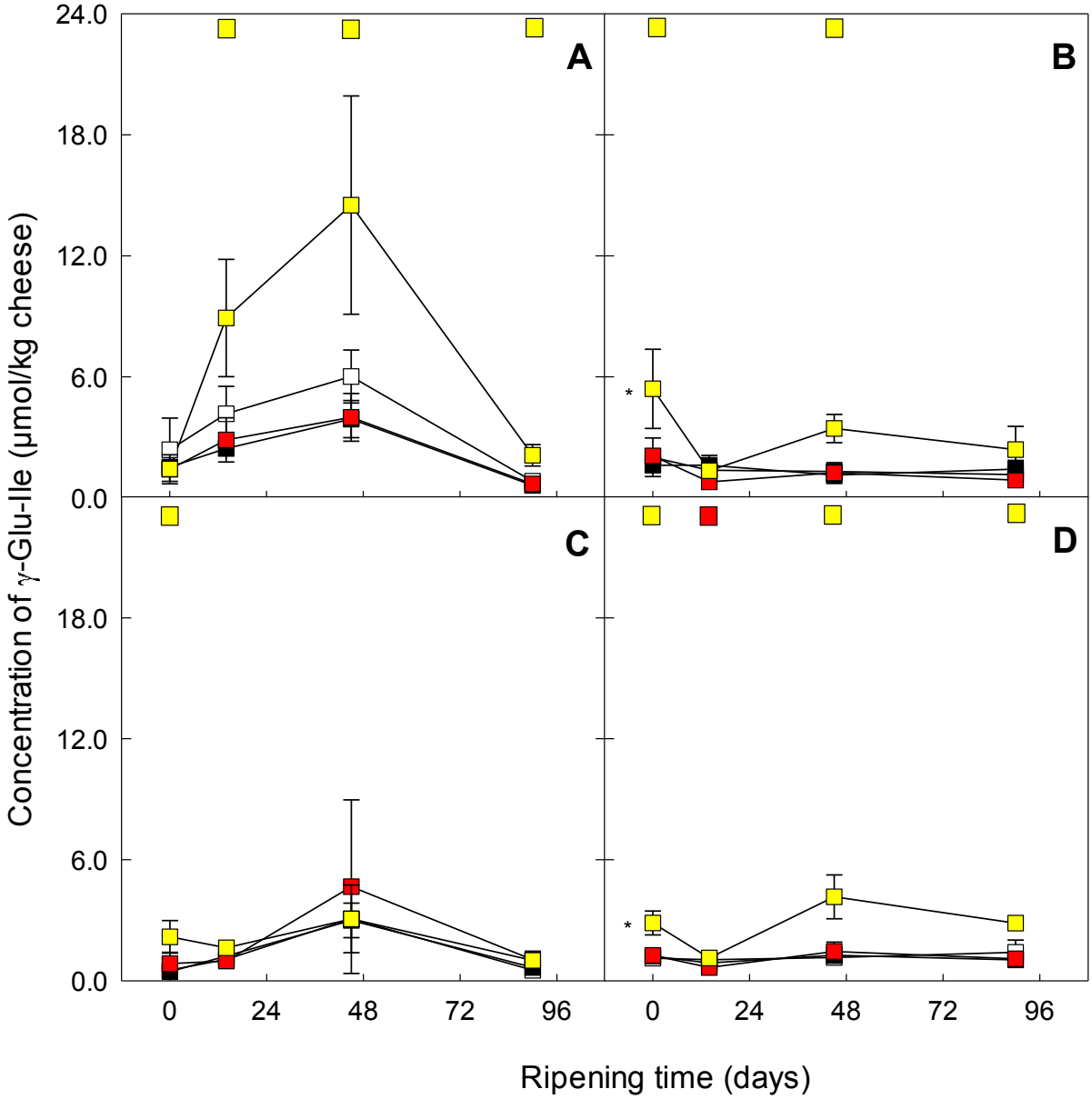



**Figure 6.5.** Concentration of γ-Glu-Ile in soy cheeses from ungerminated seeds (A) or seeds germinated: with LAB only (B), or LAB with *B. velezensis* (C) or with *B. amyloliquefaciens* (D). Colours indicate the concentration of γ-Glu-Ile in soy cheeses inoculated without adjunct strain (white), or with *Lv. hammesii* (black), *Ff. milii* (red), and *Lt. buchneri* (yellow). The symbols on the top x-axis indicated the corresponding adjuncts were significantly different ($P$<0.05) with non-adjunct control. In unripened soy cheeses, significant differences ($P$<0.05) between cheeses from ungerminated seeds and from germinated seeds were indicated with an asterisk.

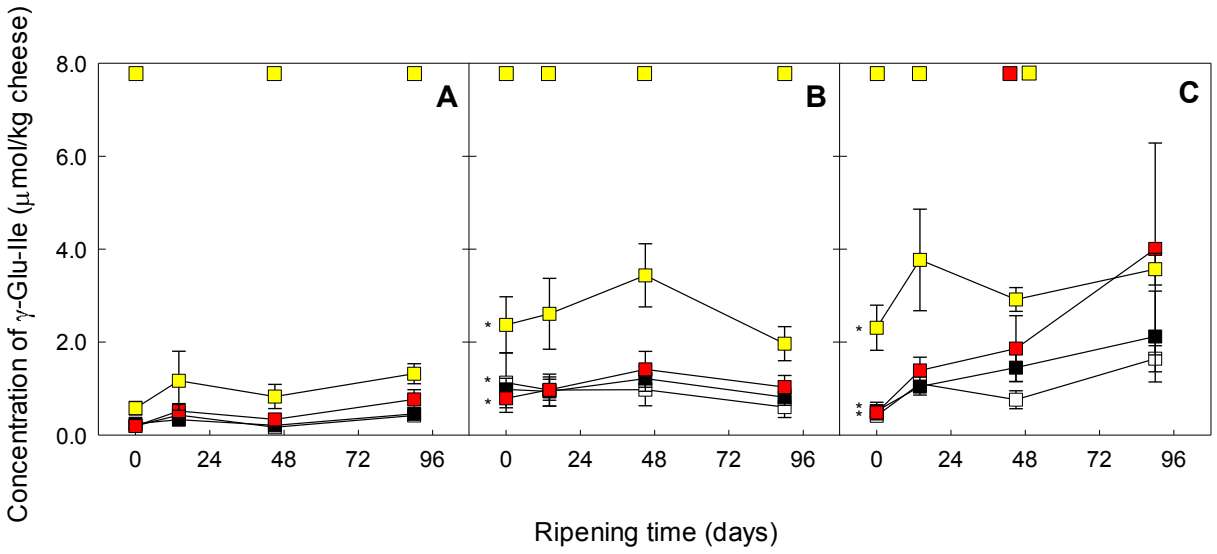



**Figure 6.6.** Concentration of γ-Glu-Ile in lupine cheeses from ungerminated seeds (A) or seeds germinated: with LAB only (B) or LAB with *B. velezensis* (C). Colours indicate the concentration of γ-Glu-Ile in lupine cheeses inoculated without adjunct strain (white), or with *Lv. hammesii* (black), *Ff. milli* (red), and *Lt. buchneri* (yellow). The symbols on the top x-axis indicated the corresponding adjuncts were significantly different ($P$<0.05) with non-adjunct control. In unripened lupine cheeses, significant differences ($P$<0.05) between cheeses from ungerminated seeds and from germinated seeds were indicated with an asterisk.

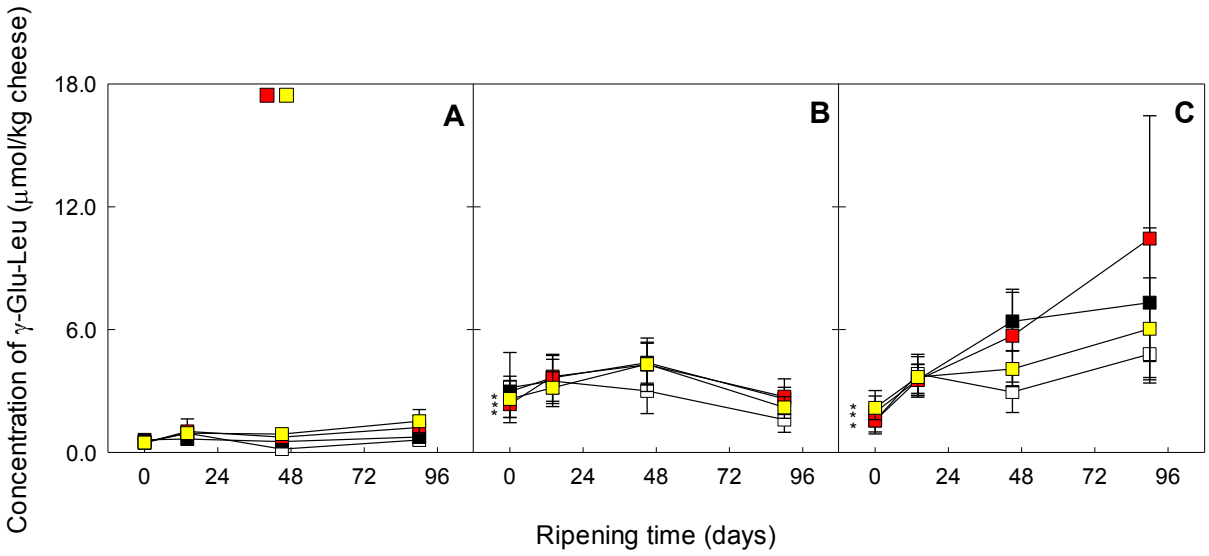



**Figure 6.7.** Concentration of γ-Glu-Leu in lupine cheeses from ungerminated seeds (A) or seeds germinated: with LAB only (B) or LAB with *B. velezensis* (C). Colours indicate the concentration of γ-Glu-Leu in lupine cheeses inoculated without adjunct strain (white), or with *Lv. hammesii* (black), *Ff. milii* (red), and *Lt. buchneri* (yellow). The symbols on the top x-axis indicated the corresponding adjuncts were significantly different ($P$<0.05) with non-adjunct control. In unripened lupine cheeses, significant differences ($P$<0.05) between cheeses from ungerminated seeds and from germinated seeds were indicated with an asterisk.

In unripened soy cheeses from germinated seeds with the adjunct culture *Ff. milii* or *Lt. buchneri*, the concentration of γ-Glu-Cys was higher ($P$<0.05) than in soy cheeses from ungerminated seeds (Figure 6.8). The soy cheeses from germinated seeds also contained higher ($P$<0.05 ) amounts of γ-Glu-Leu and γ-Glu-Ala than soy cheeses from ungerminated seeds (Figure 6.4 and Figure 6.9), but the concentration of γ-Glu-Phe was higher ($P$<0.05) in soy cheeses from ungerminated seeds when compared to soy cheeses from germinated seeds (Figure 6.9). These indicated that the seed germination impacted the concentration of γ-Glu-Cys, γ-Glu-Ala, and γ-Glu-Phe in unripened cheeses.

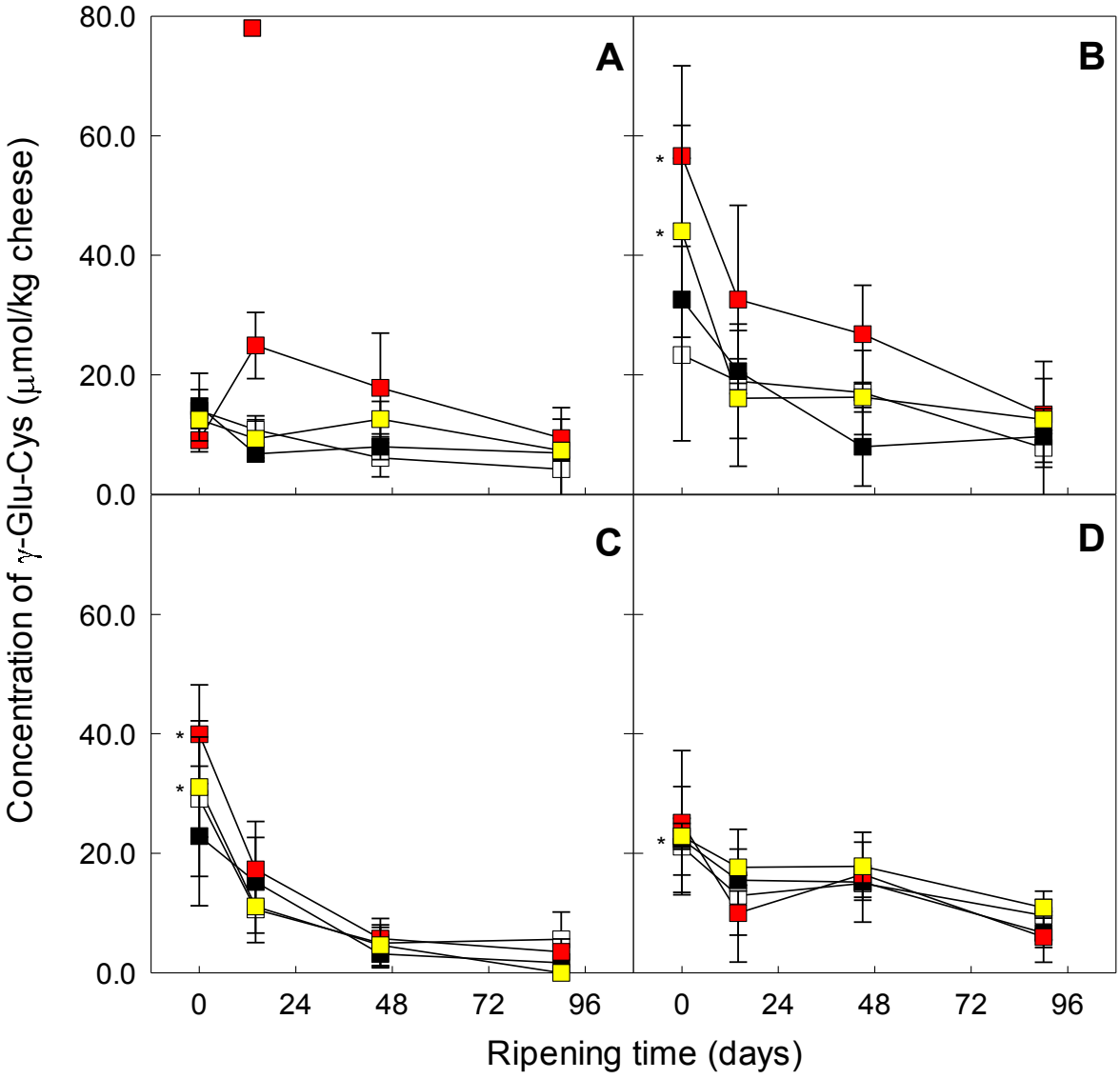



**Figure 6.8.** Concentration of γ-Glu-Cys in soy cheeses from ungerminated seeds (A) or seeds germinated: with LAB only (B), or LAB with *B. velezensis* (C) or with *B. amyloliquefaciens* (D). Colours indicate the concentration of γ-Glu-Cys in soy cheeses inoculated without adjunct strain (white), or with *Lv. hammesii* (black), *Ff. milii* (red), and *Lt. buchneri* (yellow). The symbols on the top x-axis indicated the corresponding adjuncts were significantly different ($P$<0.05) with non-adjunct control. In unripened soy cheeses, significant differences ($P$<0.05) between cheeses from ungerminated seeds and from germinated seeds were indicated with an asterisk.

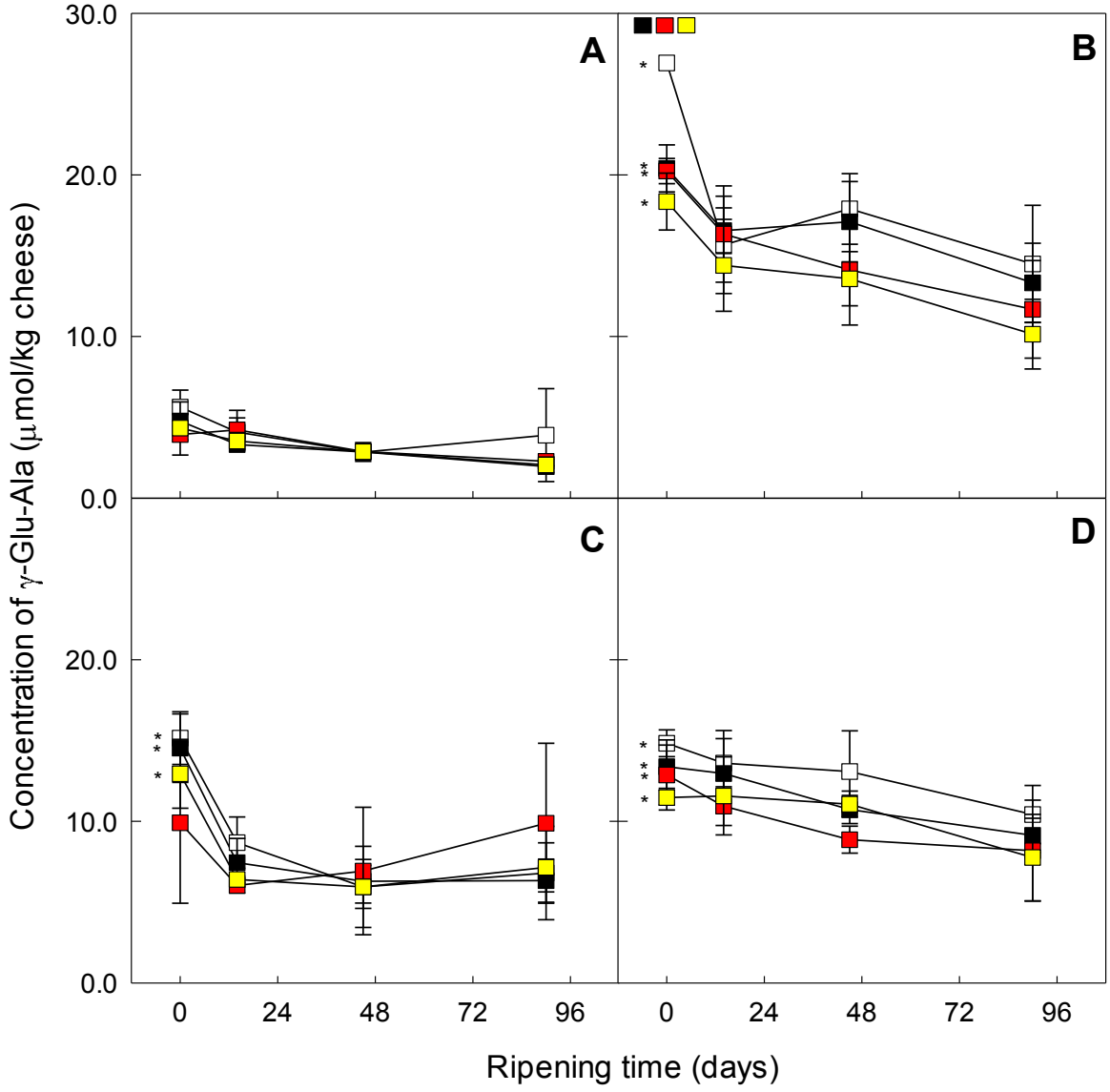



**Figure 6.9.** Concentration of γ-Glu-Ala in soy cheeses from ungerminated seeds (A) or seeds germinated: with LAB only (B), or LAB with *B. velezensis* (C) or with *B. amyloliquefaciens* (D). Colours indicate the concentration of γ-Glu-Ala in soy cheeses inoculated without adjunct strain (white), or with *Lv. hammesii* (black), *Ff. milii* (red), and *Lt. buchneri* (yellow). The symbols on the top x-axis indicated the corresponding adjuncts were significantly different ($P$<0.05) with non-adjunct control. In unripened soy cheeses, significant differences ($P$<0.05) between cheeses from ungerminated seeds and from germinated seeds were indicated with an asterisk.

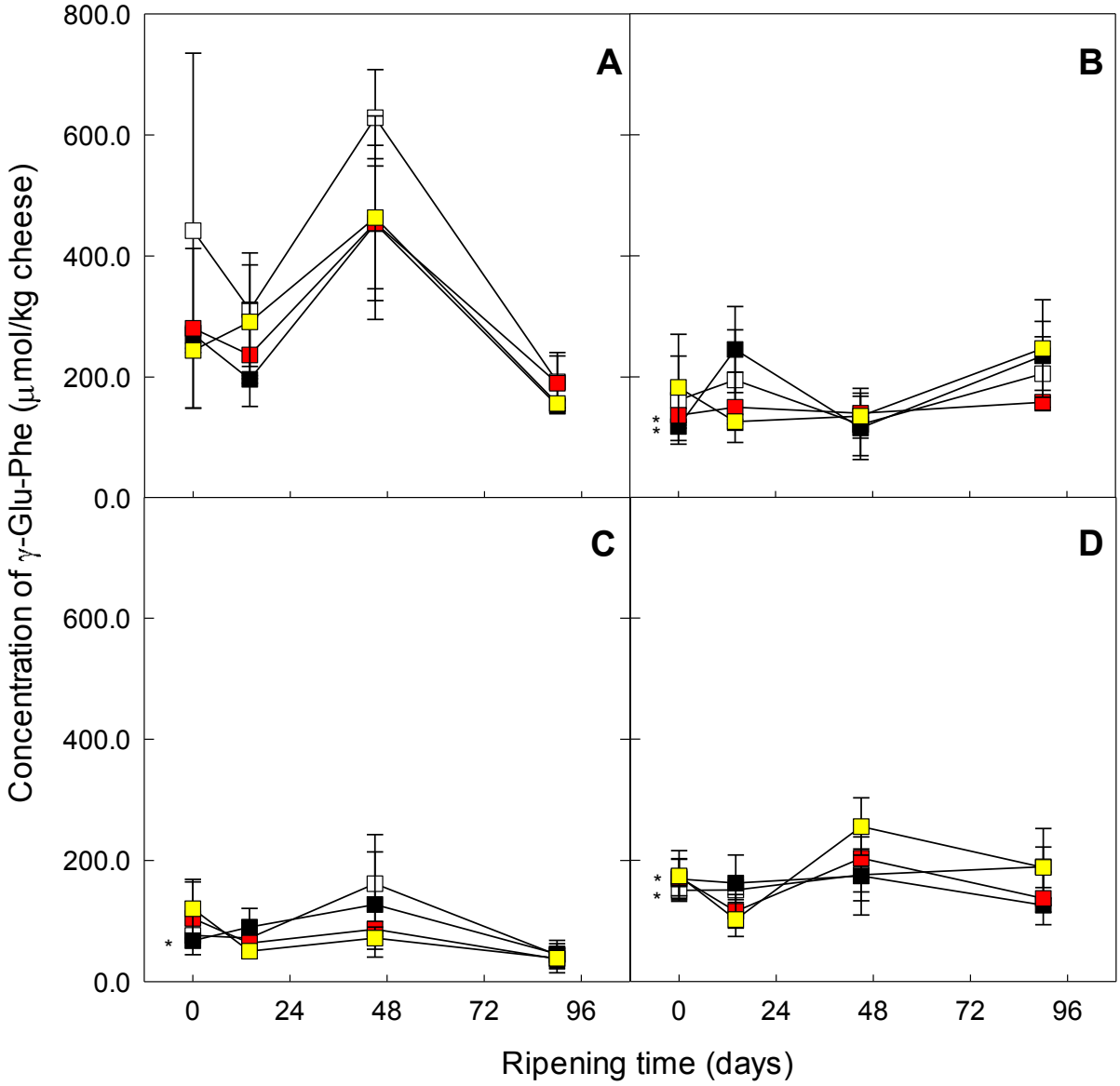



**Figure 6.10.** Concentration of γ-Glu-Phe in soy cheeses from ungerminated seeds (A) or seeds germinated: with LAB only (B), or LAB with *B. velezensis* (C) or with *B. amyloliquefaciens* (D). Colours indicate the concentration of γ-Glu-Phe in soy cheeses inoculated without adjunct strain (white), or with *Lv. hammesii* (black), *Ff. milii* (red), and *Lt. buchneri* (yellow). In unripened soy cheeses, significant differences ($P$<0.05) between cheeses from ungerminated seeds and from germinated seeds were indicated with an asterisk.

## 6.4 Discussion

A combination of culture-dependent and sequence-based methods were used to monitor the population dynamics of microbiota in plant cheeses. Sequencing of 16S rRNA amplicons from

community DNA revealed that the combination of starter cultures and adjunct cultures were the composition of the microbial community during plant cheese ripening. However, the relative abundance of viable cultures during cheese ripening were misrepresented by the DNA-based 16S rRNA gene sequencing (Wuyts et al., 2018). In samples that were ripened for one week or longer, 16 rRNA genes amplified from inactive and non-culturable cells accounted for the majority of sequences. It is thus necessary to complement DNA-based methods for characterization of (plant) cheese microbiota with a culture-based approach.

*Bacillus* spp. that produce extracellular hydrolytic enzymes occur in many fermented foods (Li et al., 2023b). In the present study, *B. velezensis* or *B. amyloliquefaciens* served as an enzyme producer during the germination of seeds which were subsequently used for production of plant cheeses. The cell counts of the *Bacillus* strains in soymilk did not differ (data not shown), however, the cell counts of *B. velezensis* during ripening were one order of magnitude higher than the cell counts of *B. amyloliquefaciens*. The stable and high cell counts obtained for both *Bacillus* strains during ripening represent spore counts rather than counts of vegetative cells and the difference between the two strains thus likely represent differences in proportion of cells that sporulated during acidification of the plant milk. The genome of *B. amyloliquefaciens* Fad 82 encodes three copies of the *spoVA*$^{2mob}$ operon while this operon is absent in *B. velezensis* FUA2155 (Li et al., 2019). The *spoVA*$^{2mob}$ operon increases heat and pressure resistance of *Bacillus* endospores but also impacts sporulation and spore germination (Krawczyk et al., 2017; Li et al., 2019).

*Lc. lactis* and *Lp. plantarum* served as starter cultures for plant milk acidification and were the dominant microbes in unripened plant cheeses. However, both strains became inactive during ripening of plant cheeses. The inactivation of *Lc. lactis* in the ripening of plant cheese from ungerminated seeds conforms to the inactivation of *Lc. lactis* as a starter culture during cheese

ripening (Parente et al., 2017). *Lp. plantarum* usually occurs in cheeses as adjunct culture that remains active with high cell counts throughout ripening (Cuesta et al., 1996; Tornadijo et al., 1995), but it was inactive as a starter culture during plant cheese ripening. In contrast, adjunct *Lv. hammesii*, *Ff. milli*, and *Lt. buchneri* survived or grew slowly during extend ripening, indicating these cultures retained metabolic activity.

In plant cheese from germinated seeds, all fermentable carbohydrates were completely depleted after 2 weeks of ripening. In plant cheese from ungerminated seeds, only galactose remained available at a concentration of 11 μmol / g (dry matter) while the concentration of galactose, raffinose, stachyose, or verbascose in plant cheese from germinated seeds was in lower than 1 μmol/g (dry matter) (data not shown). Homofermentative lactobacilli use pyruvate formate lyase as "rescue pathway" at conditions of carbohydrate starvation(Kleerebezem et al., 2020; Tanaka et al., 2002; Thomas et al., 1979). However, pyruvate formate lyase is inhibited at low pH (Gänzle, 2015; Gürtler et al., 1998), which may contribute to the poor performance of *Lp. plantarum* during cheese ripening. Heterofermentative lactobacilli convert pyruvate to acetate if electron acceptors are available, specific species also metabolize lactate to acetate and 1,2 propanediol (Gänzle, 2015). The present study documented that *Lt. buchneri* but not *Lv. hammesii* and *Ff. milli* converted lactate to acetate and 1,2-propanediol from lactate (Gänzle, 2015); in keeping with prior observations, *Lt. buchneri* FUA3252 did not further convert 1,2-propanediol to propionic acid (C. Zhang et al., 2010). The concentration of acetate generated from lactate was higher when compared to the concentration of 1,2 propanediol produced from lactate, indicating *Lt. buchneri* mainly used the oxidizing and ATP generating branch of the pathway during plant cheese ripening. In plant cheeses from germinated seeds, representing a carbohydrate depleted substrate, *Ff. milii* and *Lv. hammesii* also converted lactate via pyruvate dehydrogenase to acetate,

resulting in a lactate to acetate ratio that was substantially smaller than one (Gänzle, 2015). However, it remains unknown which metabolism pathways were used to regenerate reduced co-factors that are formed in the oxidation of lactate to acetate. The concentration of acetate and 1,2-propanediol were higher and lower, respectively, in plant cheeses from germinated seeds, suggesting that electron acceptors were generated or released during germination and may include glutathione(Jänsch et al., 2007) and hydroxycinnamic acids (Filannino et al., 2014). The concentration of acetate in 90 d ripened cheeses exceeded the odor threshold in bread 100-fold and thus likely contributes to odor and taste (Hansen and Schieberle, 2005).

The release of taste-active amino acids and peptides from plant proteins contributes to the flavor of fermented foods (Zhao et al., 2015; Zhao and Gänzle, 2016). Seed germination did not enhance the proteolysis, but the presence of *Bacillus* spp., particularly *B. velezensis* during seed germination facilitated the proteolysis in plant cheeses and the accumulation of taste-active glutamate. In addition, the proteolysis was enhanced during extend ripening. *Lc. lactis* was inactivated rapidly during plant cheese ripening. The autolysis of cells releases intracellular peptidases in cheese ripening (Lortal and Chapot-Chartier, 2005; McSweeney, 2017). The enhancement of proteolysis was thus attributed to the active proteases/peptidase produced by *Bacillus* spp. during seed germination and/or released from autolysis of *Lc. lactis* and *Lp. plantarum*.

γ-Glutamyl peptides are kokumi-active compounds. Enzymes producing these peptides in food include γ-glutamyl transpeptidase in garlic and milk (Hillmann et al., 2016; Nakamoto et al., 2018) or from *Penicillium roqueforti* (Toelstede and Hofmann, 2009) and γ-glutamyl-cysteine ligases from lactobacilli (Yan et al., 2018; Zhao and Gänzle, 2016; ). Glutaminase of *Bacillus* strains also produces γ-glutamyl peptides *in vitro* but is inactive at low pH (Yang et al., 2017).

Enzymes of seeds including both synthetic and degrading enzymes are activated during germination of plant seeds (Fernandez and Berry, 1988; Nkhata et al., 2018; G. Zhang et al., 2015). Legumes contain γ-glutamyl dipeptides and γ-glutamyl transpeptidase that synthesize γ-glutamyl dipeptides (Kasai et al., 1970; Morris and Thompson, 1962; Thompson et al., 1964). The present study documented that the concentrations of several γ-glutamyl dipeptides (γ-Glu-Leu, γ-Glu-Ile, and γ-Glu-Ala, γ-Glu-Cys) in unripened cheeses were higher after seed germination. The formation of γ-Glu-Leu, γ-Glu-Ile, γ-Glu-Ala, or γ-Glu-Cys and degradation of γ-Glu-Phe might be thus attributed to the activation of endogenous γ-glutamyl transpeptidase during seed germination (Ishikawa et al., 1967; Kasai et al., 1970). In addition, the activation of peptidase during seed germination contributed to the accumulation of γ-Glu-Cys from glutathione (Shutov and Vaintraub, 1987).

Gcl1 of *Lm. reuteri* accumulated γ-glutamyl dipeptides, particularly γ-Glu-Val, γ-Glu-Ile and γ-Glu-Leu, in sourdough fermentation (Yan et al., 2018; Zhao and Gänzle, 2016). In soy cheese fermented with lactobacilli, the accumulation of γ-Glu-Val, γ-Glu-Ile and γ-Glu-Leu showed strain-specific differences. BLAST analysis revealed that the genome of *Lv. hammesii* Lp38$^{T}$ and *Lt. buchneri* FUA3252 harbor one and four *gcl* genes, respectively, encoding for γ-glutamyl cysteine ligases (Gcls), but *gcl* was absent in the genome of *Ff. milii* FUA3115 (data not shown). Gcls *in vitro* also show high affinity to Ile and Leu (Chapter 3). In the fermentation of lupine cheese made from seeds geminated with *B. velezensis*, the accumulation of γ-Glu-Ile by *Ff. milii* was thus irrespective of Gcl activity. In contrast, the accumulation of γ-Glu-Ile or γ-Glu-Leu by *Lt. buchneri* might be attributed to Gcl activity in fermentation of soy cheeses. *Lt. buchneri* accumulated only γ-Glu-Ile in lupine cheese ripening, indicating that Gcl activity of *Lt. buchneri* was impacted by food systems.

Taken together, starter cultures *Lc. lactis* and *Lp. plantarum* were inactive during cheese ripening, but three adjunct lactobacilli retained metabolic activity throughout 45 days of ripening. Heterofermentative *Lt. buchneri* grew mainly through oxidization of lactate to acetate using ATP generating branch of pathway during cheese ripening. *Bacillus* pp. but not seed germination enhanced the proteolysis and the release of glutamate from plant cheeses. The accumulation and degradation of γ-glutamyl dipeptides were influenced by microbial enzymes and enzymes from plant seeds.

This study provides the evidence that a combination of functional starter cultures with seed germination contributes to the flavor of ripened plant cheese from whole legumes, facilitating the development of novel fermented plant cheese. These plant-based cheese analogues produced by seed germination and fermentation are rich in non-starch polysaccharides and phytochemicals including (poly)phenolic compounds. These compounds exhibit the biological activity directly or indirectly to benefit health (Armet et al., 2022; Cardona et al., 2013). The plant cheeses additionally contain high cell counts of live dietary microbes, particularly lactobacilli, which may additionally confer health benefits (Hill et al., 2023). Thus, the development of improved starter cultures for plant-based analogues of fermented dairy products may contribute to a sustainable and sustaining food supply.

# Chapter 7. General Discussion

## 7.1 Introduction

Taste-active compounds accumulate in food fermentation (Hutkins, 2019). Organic acids with sour taste are produced almost in all food fermentations with lactic acid bacteria (Gänzle, 2015). Taste-active amino acids (e.g. glutamate; hydrophobic amino acids), peptides (e.g. α-glutamyl peptides; hydrophobic peptides containing proline), or peptide derivatives (e.g. γ-glutamyl peptides; pyroglutamyl peptides) occur in many fermented foods such as sourdough bread, cheese, and soy sauce (Zhao et al., 2016). The formation of these taste-active amino acids, peptides and peptide derivatives is determined by production technology and fermentation microbiota. Although the production technology, the composition of fermentation microbiota, and the succession of microbiota differ in a diversity of food fermentations, these taste-active compounds are produced using similar pathway as follows: the primary proteolysis of raw materials by endogenous proteinases or microorganisms to generate peptides and the conversion of peptides to taste-active compounds by endogenous peptidases and metabolic activity of secondary fermentation microbes (Zhao et al., 2016). For example, in cheesemaking, peptides are generated through initial hydrolysis of caseins by proteinase originating from milk, rennet, or extracellular protease from starter culture and are subsequently converted into taste-active peptides, amino acids and peptide derivatives such as γ-glutamyl dipeptides by enzymes in milk or from starter cultures and ripening microbes (Griffiths and Tellez, 2013; Hillmann et al., 2016; McSweeney, 2017; Toelstede and Hofmann, 2009) (Figure 7.1). Some ripening microbes such as *Brevibacterium linens*, *P. roqueforti*, and *P. camemberti* can also produce potent extracellular proteinase and various peptidases to accelerate the proteolysis and the formation of taste-active

compounds in cheese ripening (McSweeney, 2017) (Figure 7.1). In traditionally soy food fermentations, extracellular hydrolytic proteases produced by mycelial fungi and/or *Bacillus* spp. decompose proteins to peptides in initial stage, followed the metabolic activity of yeasts and lactic acid bacteria to convert peptides into taste-active compounds (Chapter 2). Both enzymes in association with raw materials or production technology and metabolic activity of food-fermenting microbiota contribute to the formation of taste-active compounds in fermented foods. This PhD thesis investigated the mechanism of the formation of taste-active compounds, particularly kokumi γ-glutamyl dipeptides, in sourdough fermentation and in fermentation of plant cheese analogues made from legumes.

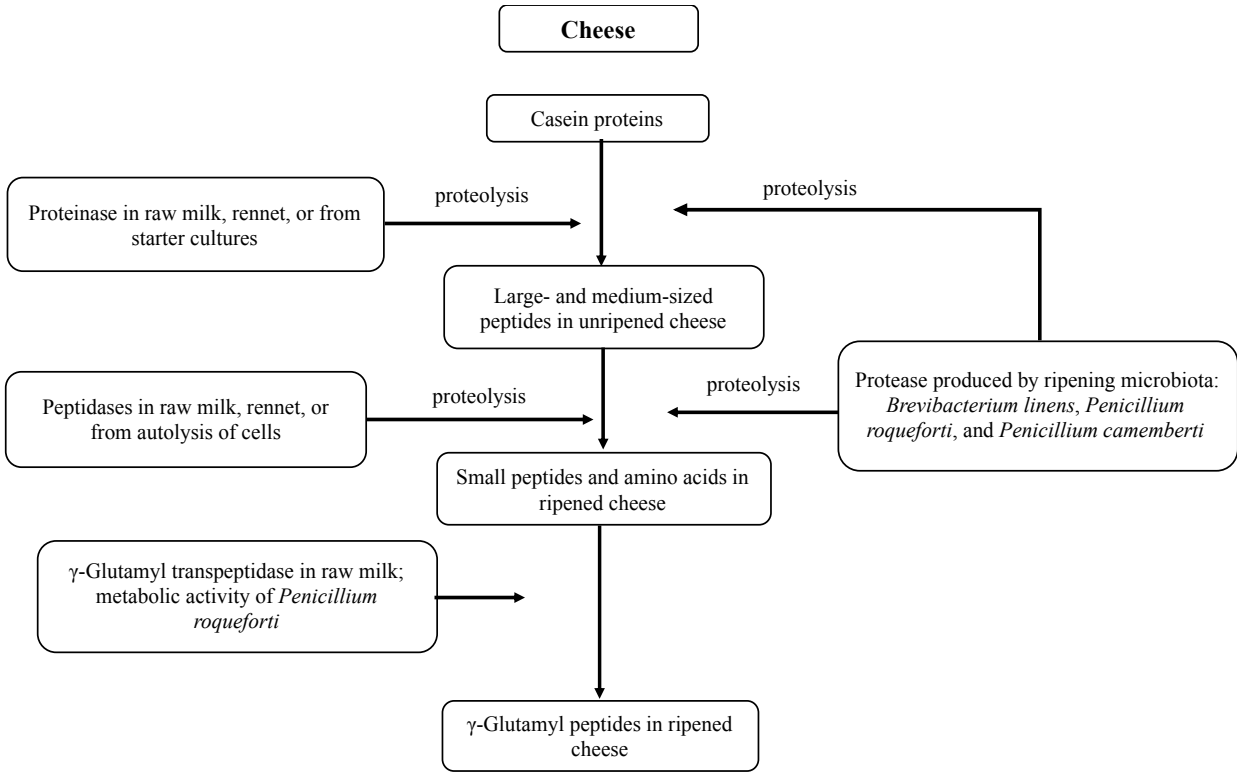



**Figure 7.1.** Overview on the formation of γ-glutamyl peptides in ripened cheese.

## 7.2 Formation of taste-active γ-glutamyl dipeptides: in sourdough or sourdough bread vs. in ripened plant cheese analogues

Sourdough is used for the bread production. Cereal proteins including wheat gluten proteins and rye secalins are important to bread flavor, volume, and texture. The degradation of cereal proteins contributes to the formation of taste-active compounds in sourdough or sourdough bread (Gänzle et al., 2008; Zhao and Gänzle, 2016). An overview on the formation of γ-glutamyl peptides in sourdough or sourdough bread is shown in Figure 7.2.

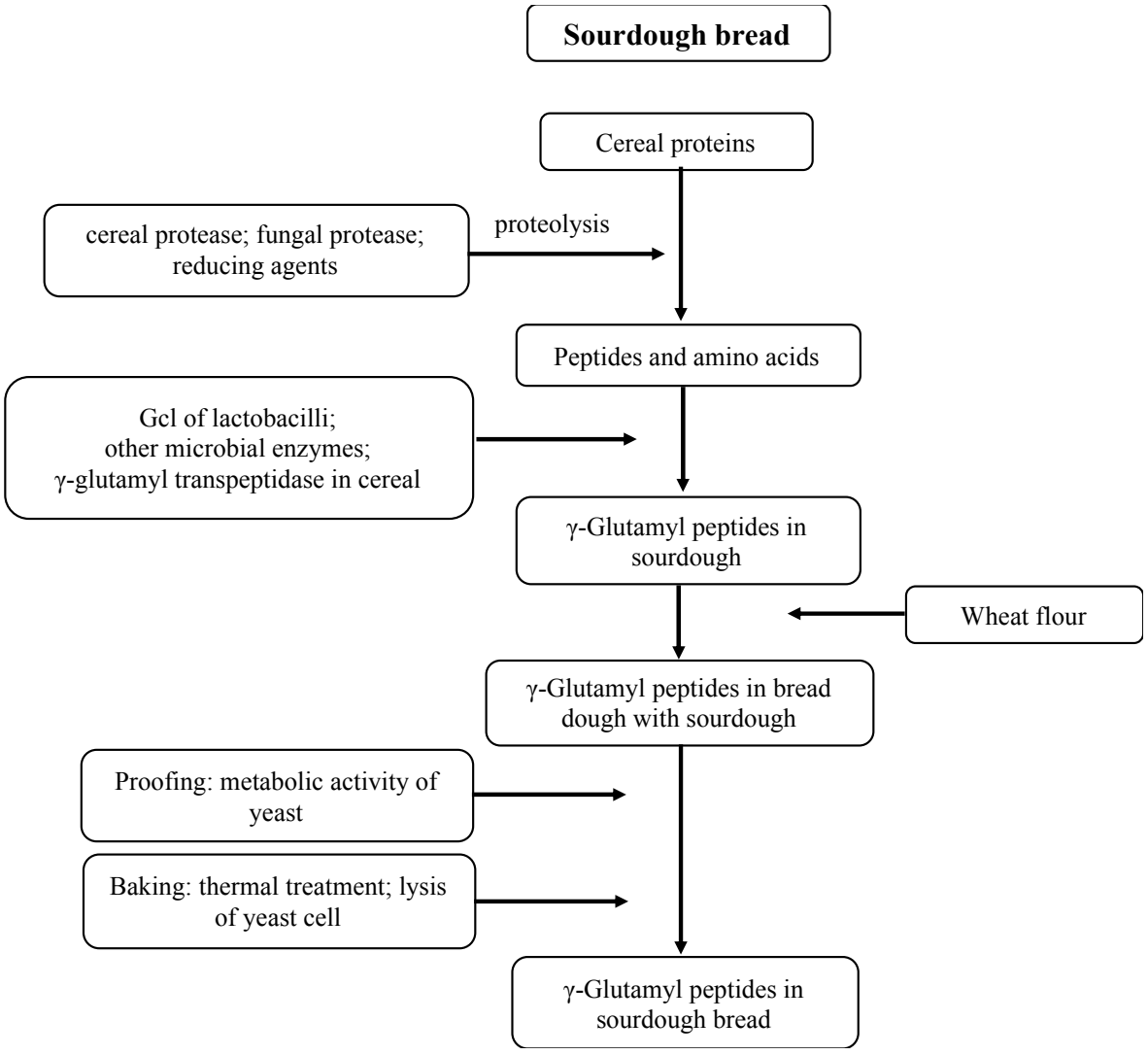



**Figure 7.2.** Overview on the formation of γ-glutamyl peptides in sourdough or sourdough bread.

Sourdough lactobacilli typically do not possess extracellular proteinase (Vermeulen et al., 2005; Zheng et al., 2015a), but acidification by lactobacilli activates proteolytic enzymes of cereals such as aspartic proteinases and carboxypeptidases to liberate peptides and amino acids from cereal proteins (Gänzle et al., 2008). The disruption of disulfide bonds of gluten by glutathione reductase of heterofermentative lactobacilli increases the substrate accessibility and accelerates proteolysis (Jänsch et al., 2007; Vermeulen et al., 2006). Fungal enzymes or reducing agents are additionally used to enhance the proteolysis of sourdough (Gänzle et al., 2008; Zhao and Gänzle, 2016). Commercial fungal proteases improved the proteolysis in sourdoughs fermented with *Lm. reuteri* strains (Chapter 3). Peptides and amino acids released by the proteolysis in sourdough support the growth of non-proteolytic lactobacilli. In addition, free amino acids additionally favor the growth of sourdough yeast (Thiele et al., 2002). These peptides and amino acids are taste-active themselves or can be converted to taste-active peptides, amino acids, or peptide derivatives by endogenous enzymes and/or metabolic activity of fermenting microbes during extensive fermentation. *Lm. reuteri* strains accumulate glutamate with umami taste from glutamine in sourdough to improve taste of bread (Zhao et al., 2015). Additionally, the accumulation of glutamate in sourdough may be related to the activity of glutaminase and glutamate decarboxylase of *Lm. reuteri* strains (Q. Li et al., 2020; Teixeira et al., 2014). A series of kokumi taste-active γ-glutamyl dipeptides accumulated in chemically acidified sourdough and sourdough fermented with *Lm. reuteri* (Chapter 4). The accumulation of γ-Glu-Ile and γ-Glu-Leu was attributed to Gcl1 activity of *Lm. reuteri*, however, the formation of other γ-glutamyl dipeptides including γ-Glu-Ala, γ-Glu-Asp, γ-Glu-Glu, γ-Glu-Cys, γ-Glu-Pro, γ-Glu-Gly, γ-Glu-Ser, and γ-Glu-Met related to the activity of enzymes in cereal and microbial enzymes other than γ-glutamyl cysteine ligases (Chapter 4). When compared to sourdough, the formation of taste-active compounds in sourdough

bread is additionally influenced by thermal treatment. Bread baking increases the concentration of kokumi GSH in the bread (Won et al., 2021). Tripeptides with two proline residues at the C-terminus, imparting bitter taste (Pripp and Ardö, 2007), are highly resistant to proteolysis but are degraded by baking (Zhao et al., 2013). Thermal treatment strongly impacted the concentration of γ-glutamyl dipeptides such as γ-Glu-Cys/Gly/Pro/Ala/Ser/Asp/Glu/Met (Chapter 4). The γ-glutamyl peptides of yeast cells (Liu et al., 2015) may be released after baking, but this remains to be investigated.

Plant cheese analogues from soy or lupine seeds predominantly consists of precipitated soy or lupine proteins (Chapter 5 and Chapter 6). The major fractions of soy or lupine proteins compose of globular 11S and 7S storage proteins. The degradation of storage proteins in seeds can be mobilized by endoproteases including acid cysteine endopeptidases, serine carboxypeptidases or neutral aminopeptidases during seed germination (Shutov and Vaintraub, 1987). However, Chapter 5 and Chapter 6 indicated endopeptidases did not participate in the degradation of storage proteins during seed germination. Soy and lupine proteins differed with respect to their degradation by proteases of *Bacillus* spp. γ-Glutamyl peptides that play an important role in the storage and metabolism of nitrogen frequently occur in plants. γ-Glu-Tyr and γ-Glu-Phe have been isolated in soy beans (Morris and Thompson, 1962). Thirteen γ-glutamyl dipeptides were identified in soy and lupine seeds/cheese and their concentration as well as their formation or hydrolysis also differed in these two substrates (Chapter 6). γ-Glutamyl peptides in plant seeds, particularly γ-Glu-Tyr and γ-Glu-Phe, are synthesized during ripening but are hydrated thereafter during seed germination (Kasai et al., 1970). γ-Glutamyl transpeptidase activated by seed germination degrades γ-Glu-Tyr or γ-Glu-Phe and simultaneously converts γ-glutamyl group generated to many other γ-glutamyl dipeptides (Ishikawa et al., 1967). The similar result was observed in

Chapter 6. It indicated that germination decreased the concentration of γ-Glu-Phe but increased the concentrations of γ-Glu-Leu, γ-Glu-Ile, γ-Glu-Ala, and γ-Glu-Cys in unripened plant cheese (Chapter 6). The hydrolytic activity of extracellular protease produced by *Bacillus* spp. accelerated the proteolysis during germination and the accumulation of taste-active glutamate (Chapter 5 and Chapter 6), which remained active throughout at least 45 d of ripening (Chapter 3). The presence of *B. velezensis* additionally increased the concentration of γ-Glu-Val in unripened soy cheese (Chapter 6). γ-Glutamyl transpeptidase generated by *B. velezensis* or activated during might contribute to the accumulation of some γ-glutamyl dipeptides such as γ-Glu-Glu/Asp/Gln/Pro during cheese ripening. The activity of intracellular protease from autolysis of starter culture LAB during cheese ripening enhanced the proteolysis of plant cheese made from ungerminated seeds or seeds germinated without bacilli was enhanced (Chapter 5). γ-Glu-Val, γ-Glu-Ile and γ-Glu-Leu were accumulated by *Lt. buchneri* but not by *Lv. hammesii* and *Ff. milii* during soy cheese ripening (Chapter 6). The accumulation of three γ-glutamyl dipeptide likely relate to Gcl activity of *Lt. buchneri*. An overview on the formation of γ-glutamyl peptides in ripened soy cheese analogues is shown in Figure 7.3.

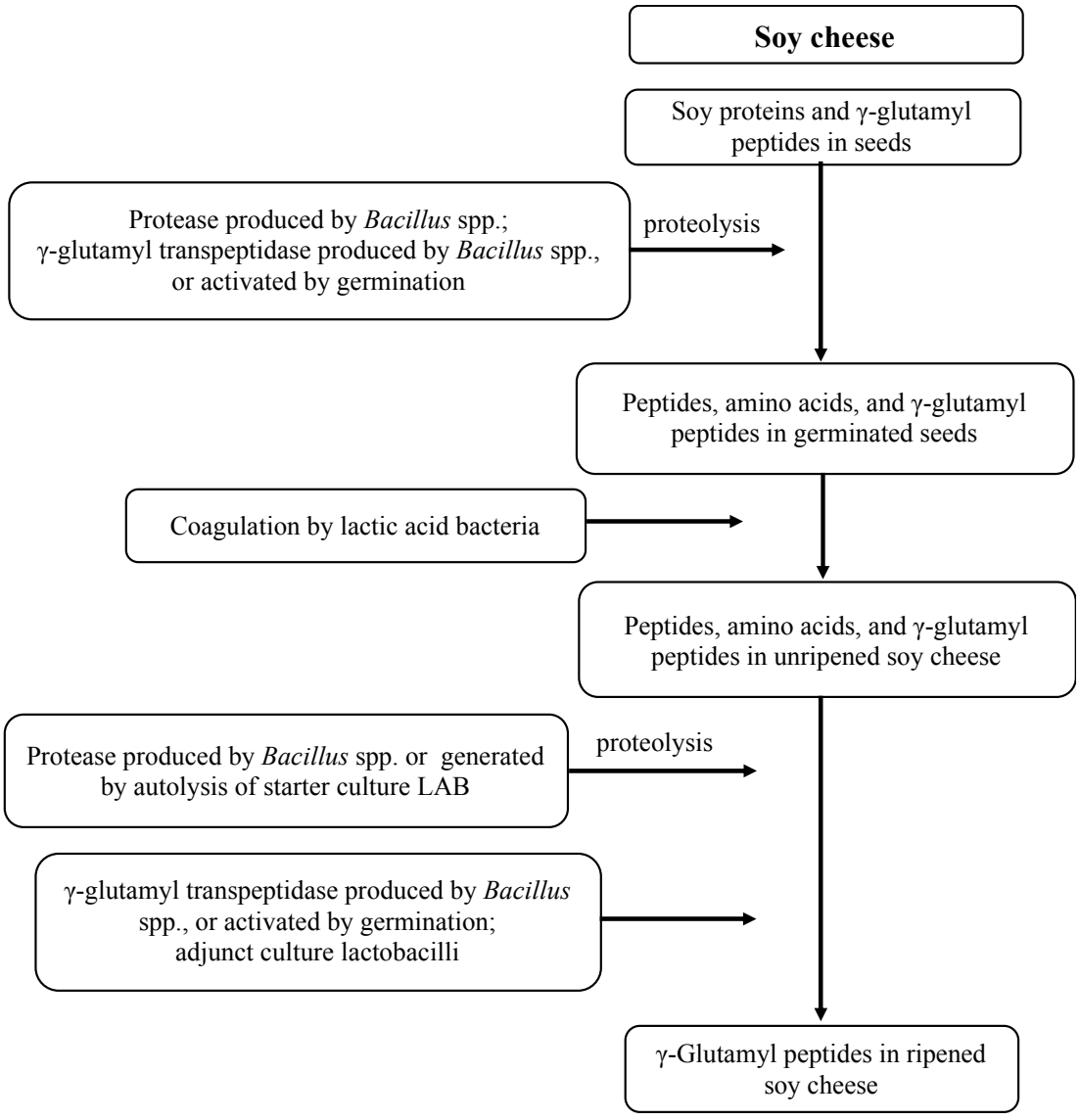



**Figure 7.3.** Overview on the formation of γ-glutamyl peptides in ripened soy cheese analogues.

Taken together, the proteolysis of sourdough or plant cheese is a key impactor for the growth of non-proteolytic lactobacilli and subsequently the formation of taste-active compounds particularly γ-glutamyl dipeptides and glutamate (Figure 7.2 and Figure 7.3). In sourdough or sourdough bread, the formation of taste-active γ-glutamyl dipeptides was attributed to the combined activity of commercial fungal protease, cereal enzymes, Gcl of lactobacilli, other microbial enzymes in lactobacilli, baker's yeast and baking process (Figure 7.2). In contrast, the

combination of seed germination, *Bacillus* spp., starter culture *Lc. lactis* and *Lp. plantarum*, and adjunct cultures contributes to the accumulation of taste-active γ-glutamyl dipeptides in fermented plant cheese analogues (Figure 7.3).

Experiments described in this thesis revealed Gcls of lactobacilli related to the accumulation of kokumi active γ-Glu-Ile and γ-Glu-Leu both in sourdough fermentation and in the fermentation of plant-based cheese analogues, indicating that Gcls of lactobacilli likely also accumulate γ-Glu-Ile and γ-Glu-Leu in other food fermentations.

## 7.3 Practical implications, limitations and future works

Although fermentation microbiota and flavor compounds of traditional fermented soy foods in Asia are well studied using metagenomic sequencing and LC/GC-MS/MS, respectively, the relationship between fermenting microbiota and the formation of flavor compounds have not been discussed. Chapter 2 well summarized the microbiology of five fermented soy foods and discuss the potential contribution of microbiota to the flavor of final products, providing the scientific guidance for the development of new plant-based cheese analogues. The combination of LAB cultures used in tempeh-making and bacilli occurring in the fermentation of *natto*, *douchi*, and *sufu* has also been successfully used in the fermentation of plant-based cheese analogues (Chapter 5 and Chapter 6), which inhibited the growth of plant microbiota including plant-associated *Enterobacteriaceae* and bacilli during seed germination and enhanced the proteolysis of plant seeds. The fate of pathogens including *Salmonella* and fungi during seed germination and production of plant cheeses, however, remains to be investigated. Taste active compounds from proteolysis during food fermentations, including taste-active amino acids, peptides and γ-glutamyl peptides are major tastants in several fermented foods such as sourdough bread and cheese. The mechanism of formation of taste active γ-glutamyl dipeptides in sourdough fermentation,

sourdough bread, and the fermentation of plant cheese analogues were studied in this thesis. The results obtained can be used to improve the control of formation of taste active compounds in food fermentations including novel plant-based foods. Nevertheless, the mechanism of formation of γ-glutamyl dipeptides is not fully understood. It is still unclear which enzymes in plant materials and from lactobacilli rather than Gcls contribute to the formation of several γ-glutamyl dipeptides. Several kokumi active γ-glutamyl dipeptides are synthesized during food fermentation, but γ-glutamyl dipeptides in final products are strongly influenced by down-stream food processing such as bread baking which can decrease or increase the concentrations of several γ-glutamyl dipeptides in bread. In addition, the kokumi threshold of γ-glutamyl peptides in bread or plant cheese analogues remains unknown. It is extremely difficult to create an appropriate food model for sensory evaluation of γ-glutamyl peptides in taste of bread or plant cheese analogues. In my study, the bread model consisting of wheat starch and skim milk powder is relatively clean with γ-glutamyl peptides, but the strong milk flavor interferes the sensory evaluation of bread model.

# BIBLIOGRAPHY

Abbott, J.J., Pei, J., Ford, J.L., Qi, Y., Grishin, V.N., Pitcher, L.A., Phillips, M.A., Grishin, N. V., 2001. Structure prediction and active site analysis of the metal binding determinants in γ-glutamylcysteine synthetase. J. Biol. Chem. 276, 42099–42107. https://doi.org/10.1074/jbc.M104672200

Abeijón Mukdsi, M.C., Falentin, H., Maillard, M.B., Chuat, V., Beatriz Medina, R., Parayre, S., Thierry, A., 2014. The secreted esterase of *Propionibacterium freudenreichii* has a major role in cheese lipolysis. Appl. Environ. Microbiol. 80, 751–756. https://doi.org/10.1128/AEM.03640-13

Abiru, Y., Ueno, T., Uchiyama, S., 2013. Isolation and characterization of novel S-equol-producing bacteria from brines of stinky tofu, a traditional fermented soy food in Taiwan. Int. J. Food Sci. Nutr. 64, 936–943. https://doi.org/10.3109/09637486.2013.816936

Albino, A., De Angelis, A., Marco, S., Severino, V., Chambery, A., Di Maro, A., Desiderio, D., Raimo, G., Masullo, M., De Vendittis, E., 2014. The cold-adapted γ-glutamyl-cysteine ligase from the psychrophile *Pseudoalteromonas haloplanktis*. Biochimie 104, 50–60. https://doi.org/10.1016/j.biochi.2014.05.003

Amin, M.N.G., Kusnadi, J., Hsu, J.L., Doerksen, R.J., Huang, T.C., 2020. Identification of a novel umami peptide in tempeh (Indonesian fermented soybean) and its binding mechanism to the umami receptor T1R. Food Chem. 333, 1274111. https://doi.org/10.1016/j.foodchem.2020.127411

André, S., Vallaeys, T., Planchon, S., 2017. Spore-forming bacteria responsible for food spoilage. Res. Microbiol. 168, 379–387. https://doi.org/10.1016/j.resmic.2016.10.003

Angelov, A., Gotcheva, V., Kuncheva, R., Hristozova, T., 2006. Development of a new oat-based probiotic drink. Int. J. Food Microbiol. 112, 75–80. https://doi.org/10.1016/j.ijfoodmicro.2006.05.015

Ariefdjohan, M.W., Nelson, P.E., Singh, R.K., Bhunia, A.K., Balasubramaniam, V.M., Singh, N., 2006. Efficacy of high hydrostatic pressure treatment in reducing *Escherichia coli* O157 and *Listeria monocytogenes* in alfalfa seeds. J. Food Sci. 69, M117–M120. https://doi.org/10.1111/j.1365-2621.2004.tb10718.x

Armet, A.M., Deehan, E.C., O'Sullivan, A.F., Mota, J.F., Field, C.J., Prado, C.M., Lucey, A.J., Walter, J., 2022. Rethinking healthy eating in light of the gut microbiome. Cell Host Microbe 30, 764–785. https://doi.org/10.1016/J.CHOM.2022.04.016

Arranz-Otaegui, A., Carretero, L.G., Ramsey, M.N., Fuller, D.Q., Richter, T., 2018. Archaeobotanical evidence reveals the origins of bread 14,400 years ago in northeastern Jordan. Proc. Natl. Acad. Sci. U. S. A. 115, 7925–7930. https://doi.org/10.1073/pnas.1801071115

Ashaolu, T.J., Reale, A., 2020. A holistic review on Euro-Asian lactic acid bacteria fermented cereals and vegetables. Microorganisms 8, 1176. https://doi.org/10.3390/MICROORGANISMS8081176

Ashenafi, M., Busse, M., 1991a. Growth of *Staphylococcus aureus* in fermenting tempeh made from various beans and its inhibition by *Lactobacillus plantarum*. J. Appl. Bacteriol. 70, 329–333. https://doi.org/10.1111/j.1365-2621.1992.tb01182.x

Ashenafi, M., Busse, M., 1991b. Development of microorganisms during cold storage of pea and chickpea tempeh and effect of *Lactobacillus plantarumn* storage microflora. J. Sci. Food

Agric. 56, 71–78. https://doi.org/10.1002/jsfa.2740560108

Bao, W., Huang, X., Liu, J., Han, B., Chen, J., 2020. Influence of *Lactobacillus brevis* on metabolite changes in bacteria-fermented sufu. J. Food Sci. 85, 165–172. https://doi.org/10.1111/1750-3841.14968

Beksan, E., Schieberle, P., Robert, F., Blank, I., Fay, L.B., Schlichtherle-Cerny, H., Hofmann, H., 2003. Synthesis and sensory characterization of novel umami-tasting glutamate glycoconjugates. J. Agric. Food Chem. 51, 5428–5436. https://doi.org/10.1021/jf0344441

Ben-Harb, S., Irlinger, F., Saint-Eve, A., Panouillé, M., Souchon, I., Bonnarme, P., 2020. Versatility of microbial consortia and sensory properties induced by the composition of different milk and pea protein-based gels. Lwt 118, 108720. https://doi.org/10.1016/j.lwt.2019.108720

Berendsen, E.M., Koning, R.A.A., Boekhorst, J., de Jong, A., Kuipers, O.P.P., Wells-Bennik, M.H.J.H.J., 2016. High-level heat resistance of spores of *Bacillus amyloliquefaciens* and *Bacillus licheniformis* results from the presence of a spoVA operon in a Tn1546 transposon. Front. Microbiol. 7, 1912. https://doi.org/10.3389/fmicb.2016.01912

Berger, C., Khan, J.A., Molimard, P., Martin, N., Spinnler, H.E., 1999. Production of sulfur flavors by ten strains of *Geotrichum candidum*. Appl. Environ. Microbiol. 65, 5510–5514. https://doi.org/10.1128/aem.65.12.5510-5514.1999

Bersaneti, G.T., Pan, N.C., Baldo, C., Celligoi, M.A.P.C., 2018. Co-production of Fructooligosaccharides and Levan by Levansucrase from *Bacillus subtilis* natto with Potential Application in the Food Industry. Appl. Biochem. Biotechnol. 184, 838–851. https://doi.org/10.1007/s12010-017-2587-0

Black, B.A., Zannini, E., Curtis, J.M., Gänzle, M.G., 2013. Antifungal hydroxy fatty acids produced during sourdough fermentation: Microbial and enzymatic pathways, and antifungal activity in bread. Appl. Environ. Microbiol. 79, 1866–1873. https://doi.org/10.1128/AEM.03784-12

Brennan, S.C., Davies, T.S., Schepelmann, M., Riccardi, D., 2014. Emerging roles of the extracellular calcium-sensing receptor in nutrient sensing: Control of taste modulation and intestinal hormone secretion. Br. J. Nutr. 111, S16–S22. https://doi.org/10.1017/S0007114513002250

Budryn, G., Klewicka, E., Grzelczyk, J., Gałązka-Czarnecka, I., Mostowski, R., 2019. Lactic acid fermentation of legume seed sprouts as a method of increasing the content of isoflavones and reducing microbial contamination. Food Chem. 285, 478–484. https://doi.org/10.1016/j.foodchem.2019.01.178

Cardona, F., Andrés-Lacueva, C., Tulipani, S., Tinahones, F.J., Queipo-Ortuño, M.I., 2013. Benefits of polyphenols on gut microbiota and implications in human health. J. Nutr. Biochem. 24, 1415–1422. https://doi.org/10.1016/j.jnutbio.2013.05.001

Chao, S.H., Sasamoto, M., Kudo, Y., Fujimoto, J., Tsai, Y.C., Watanabe, K., 2010. *Lactobacillus odoratitofui* sp. nov., isolated from stinky tofu brine. Int. J. Syst. Evol. Microbiol. 60, 2903–2907. https://doi.org/10.1099/ijs.0.019307-0

Chao, S.H., Tomii, Y., Sasamoto, M., Fujimoto, J., Tsai, Y.C., Watanabe, K., 2008a. *Lactobacillus capillatus* sp. nov., a motile bacterium isolated from stinky tofu brine. Int. J. Syst. Evol. Microbiol. 58, 2555–2559. https://doi.org/10.1099/ijs.0.65834-0

Chao, S.H., Tomii, Y., Watanabe, K., Tsai, Y.C., 2008b. Diversity of lactic acid bacteria in

fermented brines used to make stinky tofu. Int. J. Food Microbiol. 123, 134–141. https://doi.org/10.1016/j.ijfoodmicro.2007.12.010

Chen, J., Cheng, Y.Q., Yamaki, K., Li, L.T., 2007. Anti-α-glucosidase activity of Chinese traditionally fermented soybean (douchi). Food Chem. 103, 1091–1096. https://doi.org/10.1016/j.foodchem.2006.10.003

Chen, J.M., Al, K.F., Craven, L.J., Seney, S., Coons, M., McCormick, H., Reid, G., O'connor, C., Burton, J.P., 2020. Nutritional, microbial, and allergenic changes during the fermentation of cashew 'cheese' product using a quinoa-based rejuvelac starter culture. Nutrients 12, 1–12. https://doi.org/10.3390/nu12030648

Chen, Q.-C., Xu, Y.X., Wu, P., Xu, X.-Y., Pan, S.Y., 2011. Aroma impact compounds in Liuyang douchi, a Chinese traditional fermented soya bean product. Int. J. Food Sci. Technol. 46, 1823–1829. https://doi.org/10.1111/j.1365-2621.2011.02687.x

Chen, T., Jiang, S., Xiong, S., Wang, M., Zhu, D., Wei, H., 2012. Application of denaturing gradient gel electrophoresis to microbial diversity analysis in Chinese douchi. J. Sci. Food Agric. 92, 2171–2176. https://doi.org/10.1002/jsfa.5604

Chen, T., Wang, M., Jiang, S., Xiong, S., Zhu, D., Wei, H., 2011a. Investigation of the microbial changes during koji-making process of Douchi by culture-dependent techniques and PCR-DGGE. Int. J. Food Sci. Technol. 46, 1878–1883. https://doi.org/10.1111/j.1365-2621.2011.02696.x

Chen, T., Wang, M., Li, S., Wu, Q., Wei, H., 2014. Molecular identification of microbial community in surface and undersurface douchi during postfermentation. J. Food Sci. 79, M653–M658. https://doi.org/10.1111/1750-3841.12417

Chen, T., Xiong, S., Jiang, S., Wang, M., Wu, Q., Wei, H., 2011b. Molecular identification of microbial community in Chinese douchi during post-fermentation process. Food Sci. Biotechnol. 20, 1633–1638. https://doi.org/10.1007/s10068-011-0225-0

Chen, Y., Li, P., He, W., Liao, L., Xia, B., Jiang, L., Liu, Y., 2022. Analysis of microbial community and the characterization of *Aspergillus flavus* in Liuyang Douchi during fermentation. LWT - Food Sci. Technol. 154, 112567. https://doi.org/10.1016/j.lwt.2021.112567

Chen, Y., Li, P., Liao, L., Qin, Y., Jiang, L., Liu, Y., 2021. Characteristic fingerprints and volatile flavor compound variations in Liuyang Douchi during fermentation via HS-GC-IMS and HS-SPME-GC-MS. Food Chem. 361, 130055. https://doi.org/10.1016/j.foodchem.2021.130055

Cheng, Y.Q., Zhu, Y.P., Hu, Q., Li, L.T., Saito, M., Zhang, S.X., Yin, L.J., 2011. Transformation of isoflavones during sufu (a traditional Chinese fermented soybean curd) production by fermentation with *Mucor flavus* at low temperature. Int. J. Food Prop. 14, 629–639. https://doi.org/10.1080/10942910903312486

Chou, C.C., Hwan, C.H., 1994. Effect of ethanol on the hydrolysis of protein and lipid during the ageing of a chinese fermented soya bean curd—Sufu. J. Sci. Food Agric. 66, 393–398. https://doi.org/10.1002/jsfa.2740660318

Chung, H.Y., 2000. Volatile flavor components in red fermented soybean (*Glycine max*) curds. J. Agric. Food Chem. 48, 1803–1809. https://doi.org/10.1021/jf991272s

Chung, H.Y., Fung, P.K., Kim, J.S., 2005. Aroma impact components in commercial plain sufu. J. Agric. Food Chem. 53, 1684–1691. https://doi.org/10.1021/jf048617d

Comasio, A., Kerrebroeck, S.V., Vuyst, L.D., 2021. Lemon juice and apple juice used as source

of citrate and malate, respectively, enhance the formation of buttery aroma compounds and/or organic acids during Type 2 and Type 3 sourdough productions performed with *Companilactobacillus crustorum* LMG 2. Int. J. Food Microbiol. 339, 109020. https://doi.org/10.1016/J.IJFOODMICRO.2020.109020

Corzo, G., Revah, S., 1999. Production and characteristics of the lipase from *Yarrowia lipolytica* 681. Bioresour. Technol. 70, 173–180. https://doi.org/10.1016/S0960-8524(99)00024-3

Cotter, Paul D., Beresford, T.P., 2017. Microbiome changes during ripening, in: McSweeney, P.H., Fox, P.F., Cotter, P.D., Everett, D.W. (Eds.), Cheese: Chemistry, Physics and Microbiology. Elsevier Itd, London, pp. 389–409. https://doi.org/10.1016/B978-0-12-417012-4.00015-6

Cuesta, P., Fernández-García, E., De Llano, D.G., Montilla, A., Rodríguez, A., 1996. Evolution of the Microbiological and Biochemical Characteristics of Afuega'l Pitu Cheese during Ripening. J. Dairy Sci. 79, 1693–1698. https://doi.org/10.3168/jds.S0022-0302(96)76534-7

Dechet, A.M., Herman, K.M., Chen Parker, C., Taormina, P., Johanson, J., Tauxe, R. V., Mahon, B.E., 2014. Outbreaks caused by sprouts, United States, 1998-2010: Lessons learned and solutions needed. Foodborne Pathog. Dis. 11, 635–644. https://doi.org/10.1089/fpd.2013.1705

Deepankumar, S., Karthi, M., Vasanth, K., Selvakumar, S., 2019. Insights on modulators in perception of taste modalities: A review. Nutr. Res. Rev. 2, 231–246. https://doi.org/10.1017/S0954422419000118

Dias, B., Weimer, B., 1998. Conversion of methionine to thiols by lactococci, lactobacilli, and brevibacteria. Appl. Environ. Microbiol. 64, 3320–3326. https://doi.org/10.1128/aem.64.9.3320-3326.1998

Duar, R.M., Lin, X.B., Zheng, J., Martino, M.E., Grenier, T., Pérez-Muñoz, M.E., Leulier, F., Gänzle, M., Walter, J., 2017. Lifestyles in transition: evolution and natural history of the genus *Lactobacillus*. FEMS Microbiol. Rev. 41, S27–S48. https://doi.org/10.1093/femsre/fux030

Dunkel, A., Köster, J., Hofmann, T., 2007. Molecular and sensory characterization of γ-glutamyl peptides as key contributors to the kokumi taste of edible beans (Phaseolus vulgaris L.). J. Agric. Food Chem. 55, 6712–6719. https://doi.org/10.1021/jf071276u

Dwidjoseputro, D., Wolf, F.T., 1970. Microbiological studies of Indonesian fermented foodstuffs. Mycopathol. Mycol. Appl. 41, 211–222. https://doi.org/https://doi.org/10.1007/BF02051099

Dzialo, M.C., Park, R., Steensels, J., Lievens, B., Verstrepen, K.J., 2017. Physiology, ecology and industrial applications of aroma formation in yeast. FEMS Microbiol. Rev. 41, S95–S128. https://doi.org/10.1093/femsre/fux031

Endo, A., Irisawa, T., Dicks, L., Tanasupawat, S., 2014. FERMENTED FOODS: Fermentations of East and Southeast Asia, in: Encyclopedia of Food Microbiology. Elsevier Ltd, pp. 846–851. https://doi.org/10.1016/B978-0-12-384730-0.00119-1

Etten, C.H.V., Hubbard, J.E., Mallan, J.M., Smith, A.K., Blessin, C.W., 1959. Amino acid composition of soybean protein fractions. J. Agric. Food Chem. 7, 129–131. https://doi.org/10.1021/jf60096a009

Fan, J., Hu, X., Tan, S., Zhang, Y., Tatsumi, E., Li, T., 2009. Isolation and characterisation of a novel angiotensin I-converting enzyme-inhibitory peptide derived from douchi, a traditional Chinese fermented soybean food. J. Sci. Food Agric. 89, 603–608. https://doi.org/10.1002/jsfa.3482

Fan, X., Liu, G., Qiao, Y., Zhang, Y., Leng, C., Chen, H., Sun, J., Feng, Z., 2019. Characterization of volatile compounds by SPME-GC-MS during the ripening of Kedong sufu, a typical Chinese traditional bacteria-fermented soybean product. J. Food Sci. 84, 2441–2448. https://doi.org/10.1111/1750-3841.14760

Fang, Y., Franke, C., Manthei, A., McMullen, L., Temelli, F., Gänzle, M.G., 2021. Effects of high-pressure carbon dioxide on microbial quality and germination of cereal grains and beans. J. Supercrit. Fluids 175, 105272. https://doi.org/10.1016/J.SUPFLU.2021.105272

Feng, X.M., Eriksson, A.R.B., Schnürer, J., 2005. Growth of lactic acid bacteria and *Rhizopus oligosporus* during barley tempeh fermentation. Int. J. Food Microbiol. 104, 249–256. https://doi.org/10.1016/j.ijfoodmicro.2005.03.005

Feng, Z., Gao, W., Ren, D., Chen, X., Li, J., 2013. Evaluation of bacterial flora during the ripening of Kedong sufu, a typical Chinese traditional bacteria-fermented soybean product. J. Sci. Food Agric. 93, 1471–1478. https://doi.org/10.1002/jsfa.5918

Fernandez, M.L., Berry, J.W., 1988. Nutritional evaluation of chickpea and germinated chickpea flours. Plant Foods Hum. Nutr. 38, 127–134. https://doi.org/10.1007/BF01091717

Filannino, P., Gobbetti, M., De Angelis, M., Di Cagno, R., 2014. Hydroxycinnamic acids used as external acceptors of electrons: An energetic advantage for strictly heterofermentative lactic acid bacteria. Appl. Environ. Microbiol. 80, 7574–7582. https://doi.org/10.1128/aem.02413-14

Fox, P.F., Guinee, T.P., Cogan, T.M., McSweeney, P.L.H., 2017. Microbiology of cheese ripening, in: Fundamentals of Cheese Science. Springer, Boston, MA, pp. 333–390. https://doi.org/10.1007/978-1-4899-7681-9_11

Frese, S.A., Benson, A.K., Tannock, G.W., Loach, D.M., Kim, J., Zhang, M., Oh, P.L., Heng, N.C.K., Patil, P.B., Juge, N., MacKenzie, D.A., Pearson, B.M., Lapidus, A., Dalin, E., Tice, H., Goltsman, E., Land, M., Hauser, L., Ivanova, N., Kyrpides, N.C., Walter, J., 2011. The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri*. PLoS Genet. 7, e1001314. https://doi.org/10.1371/journal.pgen.1001314

Fritsch, C., Vogel, R.F., Toelstede, S., 2015. Fermentation performance of lactic acid bacteria in different lupin substrates-influence and degradation ability of antinutritives and secondary plant metabolites. J. Appl. Microbiol. 119, 1075–1088. https://doi.org/10.1111/jam.12908

Fu, R.Y., Bongers, R.S., van Swam, I.I., Chen, J., Molenaar, D., Kleerebezem, M., Hugenholtz, J., Li, Y., 2006. Introducing glutathione biosynthetic capability into *Lactococcus lactis* subsp. *cremoris* NZ9000 improves the oxidative-stress resistance of the host. Metab. Eng. 8, 662–671. https://doi.org/10.1016/j.ymben.2006.07.004

Gaborit, P., Menard, A., Morgan, F., 2001. Impact of ripening strains on the typical flavour of goat cheeses. Int. Dairy J. 11, 315–325. https://doi.org/10.1016/S0958-6946(01)00061-9

Gaillard, D., Kinnamon, S.C., 2019. New evidence for fat as a primary taste quality. Acta Physiol. 226, 1–4. https://doi.org/10.1111/apha.13246

Galant, A., Preuss, M.L., Cameron, J.C., Jez, J.M., 2011. Plant glutathione biosynthesis: Diversity in biochemical regulation and reaction products. Front. Plant Sci. 2, 1–7. https://doi.org/10.3389/fpls.2011.00045

Galle, S., Schwab, C., Arendt, E., Gänzle, M., 2010. Exopolysaccharide-forming *Weissella* strains as starter cultures for sorghum and wheat sourdoughs. J. Agric. Food Chem. 58, 5834–5841. https://doi.org/10.1021/jf1002683

Ganesan, B., Weimer, B.C., 2017. Amino acid catabolism and its relationship to cheese flavor outcomes, in: Cheese: Chemistry, Physics and Microbiology. Elsevier Ltd, pp. 483–516. https://doi.org/10.1016/B978-0-12-417012-4.00019-3

Gänzle, M., 2022. The periodic table of fermented foods: limitations and opportunities. Appl. Microbiol. Biotechnol. 106, 2815–2826. https://doi.org/10.1007/S00253-022-11909-Y

Gänzle, M., 2015. Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. Curr. Opin. Food Sci. 2, 106–117. https://doi.org/10.1016/j.cofs.2015.03.001

Gänzle, M., 2014. Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiol. 37, 2–10. https://doi.org/10.1016/j.fm.2013.04.007

Gänzle, M., Follador, R., 2012. Metabolism of oligosaccharides and starch in lactobacilli: A review. Front. Microbiol. 3, 1–15. https://doi.org/10.3389/fmicb.2012.00340

Gänzle, M., Loponen, J., Gobbetti, M., 2008. Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality. Trends Food Sci. Technol. 19, 513–521. https://doi.org/10.1016/j.tifs.2008.04.002

Gänzle, M., Zheng, J., 2019. Lifestyles of sourdough lactobacilli – do they matter for microbial ecology and bread quality? Int. J. Food Microbiol. 302, 15–23. https://doi.org/10.1016/j.ijfoodmicro.2018.08.019

Gänzle, M.G., 2020. Food fermentations for improved digestibility of plant foods – an essential ex situ digestion step in agricultural societies? Curr. Opin. Food Sci. 32, 124–132. https://doi.org/10.1016/j.cofs.2020.04.002

Gänzle, M.G., Monnin, L., Zheng, J., Zhang, L., Coton, M., Sicard, D., Walter, J., 2023a. Starter

culture development and innovation for novel fermented foods. Annu. Rev. Food Sci. Technol. 15, epub ahead of print. https://doi.org/10.1146/ANNUREV-FOOD-072023-034207

Gänzle, M.G., Qiao, N., Bechtner, J., 2023b. The quest for the perfect loaf of sourdough bread continues: Novel developments for selection of sourdough starter cultures. Int. J. Food Microbiol. 407, 4–10. https://doi.org/10.1016/j.ijfoodmicro.2023.110421

Gaur, G., Gänzle, M.G., 2023. Conversion of (poly)phenolic compounds in food fermentations by lactic acid bacteria: Novel insights into metabolic pathways and functional metabolites. Curr. Res. Food Sci. 6, 100448. https://doi.org/10.1016/J.CRFS.2023.100448

Geißinger, C., Gastl, M., Becker, T., 2022. Enzymes from cereal and fusarium metabolism involved in the malting process–A review. J. Am. Soc. Brew. Chem. 80, 1–16. https://doi.org/10.1080/03610470.2021.1911272

Glabasnia, A., Hofmann, T., 2006. Sensory-directed identification of taste-active ellagitannins in American (*Quercus alba* L.) and European oak wood (*Quercus robur* L.) and quantitative analysis in bourbon whiskey and oak-matured red wines. J. Agric. Food Chem. 54, 3380–3390. https://doi.org/10.1021/jf052617b

Griffith, O.W., 1982. Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. J. Biol. Chem. 257, 13704–13712. https://doi.org/10.1016/s0021-9258(18)33504-x

Griffiths, M.W., Tellez, A.M., 2013. *Lactobacillus helveticus*: the proteolytic system. Front. Microbiol. 4, 1–9. https://doi.org/10.3389/fmicb.2013.00030

Grossmann, L., McClements, D.J., 2021. The science of plant-based foods: Approaches to create

nutritious and sustainable plant-based cheese analogs. Trends Food Sci. Technol. 118, 207–229. https://doi.org/10.1016/J.TIFS.2021.10.004

Gu, J., Liu, T., Hou, J., Pan, L., Sadiq, F.A., Yuan, L., Yang, H., He, G., 2018a. Analysis of bacterial diversity and biogenic amines content during the fermentation processing of stinky tofu. Food Res. Int. 111, 689–698. https://doi.org/10.1016/j.foodres.2018.05.065

Gu, J., Liu, T., Sadiq, F.A., Yang, H., Yuan, L., Zhang, G., He, G., 2018b. Biogenic amines content and assessment of bacterial and fungal diversity in stinky tofu – A traditional fermented soy curd. LWT - Food Sci. Technol. 88, 26–34. https://doi.org/10.1016/j.lwt.2017.08.085

Guha, S., Majumder, K., 2022. Comprehensive Review of γ-Glutamyl Peptides (γ-GPs) and Their Effect on Inflammation Concerning Cardiovascular Health. J. Agric. Food Chem. 70, 7851–7870. https://doi.org/10.1021/acs.jafc.2c01712

Guimarães, V.M., De Rezende, S.T., Moreira, M.A., De Barros, E.G., Felix, C.R., 2001. Characterization of α-galactosidases from germinating soybean seed and their use for hydrolysis of oligosaccharides. Phytochemistry 58, 67–73. https://doi.org/10.1016/S0031-9422(01)00165-0

Guinee, T.P., 2004. Salting and the role of salt in cheese. Int. J. Dairy Technol. 57, 99–109. https://doi.org/10.1111/j.1471-0307.2004.00145.x

Gürtler, M., Gänzle, M.G., Wolf, G., Hammes, W.P., 1998. Physiological diversity among strains of *Tetragenococcus halophilus*. Syst. Appl. Microbiol. https://doi.org/10.1016/S0723-2020(98)80013-1

Hachmeister, K.A., Fung, D.Y.C., 1993. Tempeh: A mold-modified indigenous fermented food made from soybeans and/or cereal grains. Crit. Rev. Microbiol. 19, 137–188.

https://doi.org/10.3109/10408419309113527

Han, B.Z., Beumer, R.R., Rombouts, F.M., Robert Nout, M.J., 2001a. Microbiological safety and quality of commercial sufu - A Chinese fermented soybean food. Food Control 12, 541–547. https://doi.org/10.1016/S0956-7135(01)00064-0

Han, B.Z., Cao, C.F., Rombouts, F.M., Nout, M.J.R., 2004a. Microbial changes during the production of Sufu - A Chinese fermented soybean food. Food Control 15, 265–270. https://doi.org/10.1016/S0956-7135(03)00066-5

Han, B.Z., Ma, Y., Rombouts, F.M., Nout, M.J.R., 1988. Effects of temperature and relative humidity on the growth of and enzyme production by *Actinomucor taiwanensis* during sufu pehtze preparation. Appl. Environ. Microbiol. 54, 688–692. https://doi.org/10.1016/S0308-8146(02)00347-3

Han, B.Z., Rombouts, F.M., Nout, M.J.R., 2004b. Amino acid profiles of sufu, a Chinese fermented soybean food. J. Food Compos. Anal. 17, 689–698. https://doi.org/10.1016/j.jfca.2003.09.012

Han, B.Z., Rombouts, F.M., Nout, M.J.R., 2001b. A Chinese fermented soybean food. Int. J. Food Microbiol. 65, 1–10. https://doi.org/10.1016/S0168-1605(00)00523-7

Hansen, A., Schieberle, P., 2005. Generation of aroma compounds during sourdough fermentation: applied and fundamental aspects. Trends Food Sci. Technol. 16, 85–94. https://doi.org/10.1016/J.TIFS.2004.03.007

Hartanti, A.T., Rahayu, G., Hidayat, I., 2015. *Rhizopus* species from fresh tempeh collected from several regions in Indonesia. HAYATI J. Biosci. 22, 136–142. https://doi.org/10.1016/j.hjb.2015.10.004

He, G., Huang, J., Liang, R., Wu, C., Zhou, R., 2016. Comparing the differences of characteristic flavour between natural maturation and starter culture for Mucor-type Douchi. Int. J. Food Sci. Technol. 51, 1252–1259. https://doi.org/10.1111/ijfs.13077

He, R.Q., Wan, P., Liu, J., Chen, D.W., 2020. Characterisation of aroma-active compounds in Guilin Huaqiao white sufu and their influence on umami aftertaste and palatability of umami solution. Food Chem. 321, 126739. https://doi.org/10.1016/j.foodchem.2020.126739

He, W., Chen, Z., Chung, H.Y., 2022. Dynamic correlations between major enzymatic activities, physicochemical properties and targeted volatile compounds in naturally fermented plain sufu during production. Food Chem. 378, 131988. https://doi.org/10.1016/j.foodchem.2021.131988

He, W., Chung, H.Y., 2020. Exploring core functional microbiota related with flavor compounds involved in the fermentation of a natural fermented plain sufu (Chinese fermented soybean curd). Food Microbiol. 90, 103408. https://doi.org/10.1016/j.fm.2019.103408

Hebert, E.M., Raya, R.R., De Giori, G.S., 2000. Nutritional requirements and nitrogen-dependent regulation of proteinase activity of *Lactobacillus helveticus* CRL 1062. Appl. Environ. Microbiol. 66, 5316–5321. https://doi.org/10.1128/AEM.66.12.5316-5321.2000

Hibi, T., Nii, H., Nakatsu, T., Kimura, A., Kato, H., Hiratake, J., Oda, J., 2004. Crystal structure of γ-glutamylcysteine synthetase: insights into the mechanism of catalysis by a key enzyme for glutathione homeostasis. Proc. Natl. Acad. Sci. 101, 15052–15057. https://doi.org/10.1073/pnas.0403277101

Hill, C., Tancredi, D.J., Cifelli, C.J., Slavin, J.L., Gahche, J., Marco, M.L., Hutkins, R., Fulgoni, V.L., Merenstein, D., Sanders, M.E., 2023. Positive health outcomes associated with live

microbe intake from foods, including fermented foods, assessed using the NHANES database. J. Nutr. 153, 1143–1149. https://doi.org/10.1016/J.TJNUT.2023.02.019

Hillmann, H., Behr, J., Ehrmann, M.A., Vogel, R.F., Hofmann, T., 2016. Formation of kokumi-enhancing γ-glutamyl dipeptides in Parmesan cheese by means of γ-glutamyltransferase activity and stable isotope double-labeling studies. J. Agric. Food Chem. 64, 1784–1793. https://doi.org/10.1021/acs.jafc.6b00113

Holzapfel, W.H., Wood, B.J.B. (eds), 2014. Lactic acid bacteria: Biodiversity and taxonomy . John Whiley & Sons, Chichester.

Hosoi, T., Kiuchi, K., 2008. Natto: A soybean food made by fermenting cooked soybeans with *Bacillus subtilis (Natto)*., in: Handbook of Fermented Functional Foods. Taylor and Francis, pp. 267–290. https://doi.org/10.1201/9780203009727-14

Hu, H., Hao, J., Cheng, Y., Yin, L., Ma, Y., Yu, Z., Li, L., 2012. Effect of fermented rice culture on the microbiological, biochemical and sensory characteristics of low-salt douchi, a traditional Chinese fermented soybean condiment. Int. J. Food Sci. Technol. 47, 689–695. https://doi.org/10.1111/j.1365-2621.2011.02894.x

Hu, W., Liu, Z., Fu, B., Zhang, X., Qi, Y., Hu, Y., Wang, C., Li, D., Xu, N., 2022. Metabolites of the soy sauce koji making with *Aspergillus niger* and *Aspergillus oryzae*. Int. J. Food Sci. Technol. 57, 301–309. https://doi.org/10.1111/ijfs.15406

Huang, X., Yu, S., Han, B., Chen, J., 2018. Bacterial community succession and metabolite changes during sufu fermentation. LWT 97, 537–545. https://doi.org/10.1016/j.lwt.2018.07.041

Hutkins, R.W., 2019. Microbiology and Technology of Fermented Foods, 2nd Edition. ed. Wiley-

Blackwell, Hoboken, New Jersey.

Ichishima, E., Takada, Y., Taira, K., Takeuchi, M., 1986. Specificities of extracellular and ribosomal serine proteinases from *Bacillus natto*, a food microorganism. Biochim. Biophys. Acta (BBA)/Protein Struct. Mol. 869, 178–184. https://doi.org/10.1016/0167-4838(86)90292-X

Irlinger, F., Helinck, S., Jany, J.L., 2017. Secondary and adjunct cultures, in: Cheese: Chemistry, Physics and Microbiology. Elsevier Ltd, pp. 273–300. https://doi.org/10.1016/B978-0-12-417012-4.00011-9

Ishikawa, Y., Hasegawa, S., Obata, Y., 1967. Changes in amino acid composition during germination of soybean. Agric. Biol. Chem. 31, 490–493. https://doi.org/10.1080/00021369.1967.10858831

Janowiak, B.E., Griffith, O.W., 2005. Glutathione synthesis in *Streptococcus agalactiae*: one protein accounts for γ-glutamylcysteine synthetase and glutathione synthetase activities. J. Biol. Chem. 280, 11829–11839. https://doi.org/10.1074/jbc.M414326200

Jänsch, A., Korakli, M., Vogel, R.F.R.F., Gänzle, M.G.M.G., 2007. Glutathione reductase from *Lactobacillus sanfranciscensis* DSM20451$^{T}$: Contribution to oxygen tolerance and thiol exchange reactions in wheat sourdoughs. Appl. Environ. Microbiol. 73, 4469–4476. https://doi.org/10.1128/AEM.02322-06

Jeleń, H., Majcher, M., Ginja, A., Kuligowski, M., 2013. Determination of compounds responsible for tempeh aroma. Food Chem. 141, 459–465. https://doi.org/10.1016/j.foodchem.2013.03.047

Jeong, M., Jeong, D.W., Lee, J.H., 2015. Safety and biotechnological properties of *Enterococcus*

*faecalis* and *Enterococcus faecium* isolates from *Meju*. J. Korean Soc. Appl. Biol. Chem. 58, 813–820. https://doi.org/10.1007/s13765-015-0110-2

Jiang, S., Cai, W., Xu, B., 2013. Food quality improvement of soy milk made from short-time germinated soybeans. Foods 2, 198–212. https://doi.org/10.3390/foods2020198

Ju, S., Cao, Z., Wong, C., Liu, Y., Foda, M.F., Zhang, Z., Li, J., 2019. Isolation and optimal fermentation condition of the *Bacillus subtilis* subsp. *Natto* strain wtc016 for nattokinase production. Fermentation 5, 1–12. https://doi.org/10.3390/fermentation5040092

Kada, S., Yabusaki, M., Kaga, T., Ashida, H., Yoshida, K.I., 2008. Identification of two major ammonia-releasing reactions involved in secondary natto fermentation. Biosci. Biotechnol. Biochem. 72, 1869–1876. https://doi.org/10.1271/bbb.80129

Kanno, A., Takamatsu, H., 1987. Changes in the volatile components of "natto" during manufacturing and storage. Nippon SHOKUHIN KOGYO GAKKAISHI 34, 330–335. https://doi.org/10.3136/nskkk1962.34.5_330

Karametsi, K., Kokkinidou, S., Ronningen, I., Peterson, D.G., 2014. Identification of bitter peptides in aged cheddar cheese. J. Agric. Food Chem. 62, 8034–8041. https://doi.org/10.1021/jf5020654

Kasai, T., Sakamura, S., Ohashi, S., Kumagai, H., 1970. Amino Acid Composition of Soybean 34, 1848–1850. https://doi.org/https://doi.org/10.1080/00021369.1970.10859858

Kelly, B.S., Antholine, W.E., Griffith, O.W., 2002. *Escherichia coli* γ-glutamylcysteine synthetase: two active site metal ions affect substrate and inhibitor binding. J. Biol. Chem. 277, 50–58. https://doi.org/10.1074/jbc.M107961200

Keuth, S., Bisping, B., 1994. Vitamin B12 production by *Citrobacter freundii* or *Klebsiella*

*pneumoniae* during tempeh fermentation and proof of enterotoxin absence by PCR. Appl. Environ. Microbiol. 60, 1495–1499. https://doi.org/10.1128/aem.60.5.1495-1499.1994

Kim, B., Byun, B.Y., Mah, J.H., 2012. Biogenic amine formation and bacterial contribution in *Natto* products. Food Chem. 135, 2005–2011. https://doi.org/10.1016/j.foodchem.2012.06.091

Kim, J.S., Lu, Y., Chung, H.Y., 2011. Chemical and textural properties in commercial fermented soybean curds of sufu. J. Food Sci. Nutr. 16, 55–61. https://doi.org/10.3746/jfn.2011.16.1.055

Kino, K., Kuratsu, S., Noguchi, A., Kokubo, M., Nakazawa, Y., Arai, T., Yagasaki, M., Kirimura, K., 2007. Novel substrate specificity of glutathione synthesis enzymes from *Streptococcus agalactiae* and *Clostridium acetobutylicum*. Biochem. Biophys. Res. Commun. 352, 351–359. https://doi.org/10.1016/j.bbrc.2006.11.016

Kleerebezem, M., Bachmann, H., van Pelt-KleinJan, E., Douwenga, S., Smid, E.J., Teusink, B., van Mastrigt, O., 2020. Lifestyle, metabolism and environmental adaptation in *Lactococcus lactis*. FEMS Microbiol. Rev. 44, 804–820. https://doi.org/10.1093/femsre/fuaa033

Kłosowski, G., Mikulski, D., Pielech-Przybylska, K., 2021. Pyrazines biosynthesis by *Bacillus* strains isolated from natto fermented soybean. Biomolecules 11, 1–12. https://doi.org/10.3390/biom11111736

Kong, M., Wang, F., Tian, L., Tang, H., Zhang, L., 2018. Functional identification of glutamate cysteine ligase and glutathione synthetase in the marine yeast *Rhodosporidium diobovatum*. Sci. Nat. 105, 1–9. https://doi.org/10.1007/s00114-017-1520-2

Kosuge, T., Zenda, H., Tsuji, K., Yamamoto, T., Narita, H., 1971. Studies on flavor components of foodstuffs distribution of tetramethylpyrazine in fermented foodstuffs. Agric. Biol. Chem.

35, 693–696. https://doi.org/10.1080/00021369.1971.10859981

Krawczyk, A.O., de Jong, A., Omony, J., Holsappel, S., Wells-Bennik, M.H.J., Kuipers, O.P., Eijlander, R.T., 2017. Spore heat activation requirements and germination responses correlate with sequences of germinant receptors and with the presence of a specific *spoVA2mob* operon in foodborne strains of *Bacillus subtilis*. Appl. Environ. Microbiol. 83, e03122-16. https://doi.org/10.1128/AEM.03122-16/FORMAT/EPUB

Kubo, Y., Rooney, A.P., Tsukakoshi, Y., Nakagawa, R., Hasegawa, H., Kimura, K., 2011. Phylogenetic analysis of *Bacillus subtilis* strains applicable to natto (fermented soybean) production. Appl. Environ. Microbiol. 77, 6463–6469. https://doi.org/10.1128/AEM.00448-11

Kumari, A., Singh, V.K., Fitter, J., Polen, T., Kayastha, A.M., 2010. α-Amylase from germinating soybean (*Glycine max*) seeds - Purification, characterization and sequential similarity of conserved and catalytic amino acid residues. Phytochemistry 71, 1657–1666. https://doi.org/10.1016/j.phytochem.2010.06.012

Kunioka, M., 1995. Biosynthesis of poly (γ-glutamic acid) from l-glutamine, citric acid and ammonium sulfate in *Bacillus subtilis* IFO3335. Appl. Microbiol. Biotechnol. 44, 501–506. https://doi.org/10.1007/BF00169951

Kuroda, M., Kato, Y., Yamazaki, J., Kai, Y., Mizukoshi, T., Miyano, H., Eto, Y., 2013. Determination and quantification of the kokumi peptide, γ-glutamyl-valyl-glycine, in commercial soy sauces. Food Chem. 141, 823–828. https://doi.org/10.1016/j.foodchem.2013.03.070

Kuroda, M., Miyamura, N., 2015. Mechanism of the perception of "*kokumi*" substances and the

sensory characteristics of the "*kokumi*" peptide, γ-Glu-Val-Gly. Flavour 4, 1–3. https://doi.org/10.1186/2044-7248-4-11

Kustyawati, M.E., Nawansih, O., Nurdjanah, S., 2017. Profile of aroma compounds and acceptability of modified tempeh. Int. Food Res. J. 24, 734–740.

Lan, G., Li, C., He, L., Zeng, X., Zhu, Q., 2020. Effects of different strains and fermentation method on nattokinase activity, biogenic amines, and sensory characteristics of natto. J. Food Sci. Technol. 57, 4414–4423. https://doi.org/10.1007/s13197-020-04478-3

Lee, J.I., Londono, M., Hirschberger, L.L., Stipanuk, M.H., 2004. Regulation of cysteine dioxygenase and γ-glutamylcysteine synthetase is associated with hepatic cysteine level. J. Nutr. Biochem. 15, 112–122. https://doi.org/10.1016/j.jnutbio.2003.10.005

Lei, X., Sun, G., Xie, J., Wei, D., 2013. *Lactobacillus curieae* sp. nov., isolated from stinky tofu brine. Int. J. Syst. Evol. Microbiol. 63, 2501–2505. https://doi.org/10.1099/ijs.0.041830-0

Li, C., Xu, T., Liu, X.W., Wang, X., Xia, T., 2021. The expression of β-glucosidase during natto fermentation increased the active isoflavone content. Food Biosci. 43, 101286. https://doi.org/10.1016/J.FBIO.2021.101286

Li, K., Tang, J., Zhang, Z., Wu, Z., Zhong, A., Li, Z., Wang, Y., 2022. Correlation between flavor compounds and microorganisms of Chaling natural fermented red sufu. LWT 154, 112873. https://doi.org/10.1016/j.lwt.2021.112873

Li, L., Ruan, L., Ji, A., Wen, Z., Chen, S., Wang, L., Wei, X., 2018. Biogenic amines analysis and microbial contribution in traditional fermented food of Douchi. Sci. Rep. 8, 1–10. https://doi.org/10.1038/s41598-018-30456-z

Li, Q., Tao, Q., Teixeira, J.S., Su, M.S., Gänzle, M.G., 2020. Contribution of glutaminases to

glutamine metabolism and acid resistance in *Lactobacillus reuteri* and other vertebrate host adapted lactobacilli. Food Microbiol. 86, 103343. https://doi.org/10.1016/j.fm.2019.103343

Li, Q., Xia, Y., Zhou, L., Xie, J., 2013. Evaluation of the rheological, textural, microstructural and sensory properties of soy cheese spreads. Food Bioprod. Process. 91, 429–439. https://doi.org/10.1016/j.fbp.2013.03.001

Li, Q., Zhang, L., Arneborg, N., Lametsch, R., 2022. Influence of growth medium and yeast species on the formation of γ-glutamyl peptides. LWT 165. https://doi.org/10.1016/j.lwt.2022.113716

Li, W., Li, Z., Yang, J., Ye, Q., 2011. Production of glutathione using a bifunctional enzyme encoded by *gshF* from *Streptococcus thermophilus* expressed in *Escherichia coli*. J. Biotechnol. 154, 261–268. https://doi.org/10.1016/j.jbiotec.2011.06.001

Li, X., He, Y., Yang, W., Mu, D., Zhang, M., Dai, Y., Zheng, Z., Jiang, S., Wu, X., 2021. Comparative analysis of the microbial community and nutritional quality of sufu. Food Sci. Nutr. 9, 4117–4126. https://doi.org/10.1002/fsn3.2372

Li, Y., Zhang, X., Yang, J.J., Ma, X.Y., Jia, X.D., Du, P., Li, A. li, 2020. Influence of the addition of Geotrichum candidum on the microbial, chemical, textural, and sensory features of soft soy cheese. J. Food Process. Preserv. 44, 1–11. https://doi.org/10.1111/jfpp.14823

Li, Y.Y., Yu, R.C., Chou, C.C., 2010. Some biochemical and physical changes during the preparation of the enzyme-ripening sufu, a fermented product of soybean curd. J. Agric. Food Chem. 58, 4888–4893. https://doi.org/10.1021/jf904600a

Li, Z., Fernandez, K.X., Vederas, J.C., Gänzle, M.G., 2023a. Composition and activity of antifungal lipopeptides produced by *Bacillus* spp. in *daqu* fermentation. Food Microbiol. 111,

104211. https://doi.org/10.1016/J.FM.2022.104211

Li, Z., Schottroff, F., Simpson, D.J., Gänzle, M.G., 2019. The copy number of the spoVA2mob operon determines pressure resistance of Bacillus endospores. Appl. Environ. Microbiol. 85. https://doi.org/10.1128/AEM.01596-19

Li, Z., Siepmann, F.B., Rojas Tovar, L.E., Chen, X., Gänzle, M., 2020. Effect of copy number of the *spoVA*$^{2mob}$ operon, sourdough and reutericyclin on ropy bread spoilage caused by *Bacillus* spp. Food Microbiol. 91, 103507. https://doi.org/10.1016/j.fm.2020.103507

Li, Z., Zheng, M., Zheng, J., Gänzle, M.G., 2023b. *Bacillus* species in food fermentations: an underappreciated group of organisms for safe use in food fermentations. Curr. Opin. Food Sci. 50, 101007. https://doi.org/10.1016/J.COFS.2023.101007

Liang, J., Li, D., Shi, R., Wang, J., Guo, S., Ma, Y., Xiong, K., 2019. Effects of microbial community succession on volatile profiles and biogenic amine during sufu fermentation. LWT 114, 108379. https://doi.org/10.1016/j.lwt.2019.108379

Liao, D., Cram, D., Sharpe, A.G., Marsolais, F., 2013. Transcriptome profiling identifies candidate genes associated with the accumulation of distinct sulfur γ-glutamyl dipeptides in *Phaseolus vulgaris* and *Vigna mungo* seeds. Front. Plant Sci. 4, 1–12. https://doi.org/10.3389/fpls.2013.00060

Lie, A., Braconi, N., Boom, R.M., van der Padt, A., 2021. Less refined ingredients have lower environmental impact – A life cycle assessment of protein-rich ingredients from oil- and starch-bearing crops. J. Clean. Prod. 292, 126046. https://doi.org/10.1016/j.jclepro.2021.126046

Lin, J., Xie, J., Luo, L., Gänzle, M., 2023. Characterization of GshAB of Tetragenococcus

halophilus: a two-domain glutathione synthetase. Appl. Microbiol. Biotechnol. 107, 2997–3008. https://doi.org/10.1007/s00253-023-12497-1

Lite, L., 2005. Asian fermented soybean products, in: Handbook of Food Science, Technology, and Engineering. CRC Press, pp. 355–374. https://doi.org/10.1201/b15995-26

Lite, L., Lijun, Y., Jianhua, Z., Xiaofeng, Z., Lin, Z., 2002. Functionalities of traditional foods in China. 9th J/RCAS Int. Symp. 140–144.

Liu, C.J., Gong, F.M., Li, X.R., Li, H.Y., Zhang, Z.H., Feng, Y., Nagano, H., 2012. Natural populations of lactic acid bacteria in douchi from Yunnan Province, China. J. Zhejiang Univ. Sci. B 13, 298–306. https://doi.org/10.1631/jzus.b1100221

Liu, G., Liu, Y., Ro, K.S., Du, L., Tang, Y.J., Zhao, L., Xie, J., Wei, D., 2022. Genomic characteristics of a novel strain *Lactiplantibacillus plantarum* X7021 isolated from the brine of stinky tofu for the application in food fermentation. LWT 156, 113054. https://doi.org/10.1016/j.lwt.2021.113054

Liu, J., Song, H., Liu, Y., Li, P., Yao, J., Xiong, J., 2015. Discovery of kokumi peptide from yeast extract by LC-Q-TOF-MS/MS and sensomics approach. J. Sci. Food Agric. 95, 3183–3194. https://doi.org/10.1002/jsfa.7058

Liu, L., Chen, X., Hao, L., Zhang, G., Jin, Z., Li, C., Yang, Y., Rao, J., Chen, B., 2022. Traditional fermented soybean products: processing, flavor formation, nutritional and biological activities. Crit. Rev. Food Sci. Nutr. 62, 1971–1989. https://doi.org/10.1080/10408398.2020.1848792

Liu, Y., Song, H.L., Luo, H., 2018a. Correlation between the key aroma compounds and gDNA copies of *Bacillus* during fermentation and maturation of natto. Food Res. Int. 112, 175–183.

https://doi.org/10.1016/j.foodres.2018.06.033

Liu, Y., Su, H., Song, H.L., 2018b. Comparison of four extraction methods, SPME, DHS, SAFE, versus SDE, for the analysis of flavor compounds in natto. Food Anal. Methods 11, 343–354. https://doi.org/10.1007/s12161-017-1005-0

Lo, R., Ho, V.T.T., Bansal, N., Turner, M.S., 2018. The genetic basis underlying variation in production of the flavour compound diacetyl by *Lactobacillus rhamnosus* strains in milk. Int. J. Food Microbiol. 265, 30–39. https://doi.org/10.1016/J.IJFOODMICRO.2017.10.029

Lo, R., Turner, M.S., Barry, D.G., Sreekumar, R., Walsh, T.P., Giffard, P.M., 2009. Cystathionine γ-lyase is a component of cystine-mediated oxidative defense in *Lactobacillus reuteri* BR11. J. Bacteriol. 191, 1827–1837. https://doi.org/10.1128/JB.01553-08

Lortal, S., Chapot-Chartier, M.P., 2005. Role, mechanisms and control of lactic acid bacteria lysis in cheese. Int. Dairy J. 15, 857–871. https://doi.org/10.1016/J.IDAIRYJ.2004.08.024

Marco, M.L., Sanders, M.E., Gänzle, M., Arrieta, M.C., Cotter, P.D., De Vuyst, L., Hill, C., Holzapfel, W., Lebeer, S., Merenstein, D., Reid, G., Wolfe, B.E., Hutkins, R., 2021. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on fermented foods. Nat. Rev. Gastroenterol. Hepatol. 18, 196–208. https://doi.org/10.1038/s41575-020-00390-5

Martino, M.E., Bayjanov, J.R., Caffrey, B.E., Wels, M., Joncour, P., Hughes, S., Gillet, B., Kleerebezem, M., van Hijum, S.A.F.T., Leulier, F., 2016. Nomadic lifestyle of *Lactobacillus plantarum* revealed by comparative genomics of 54 strains isolated from different habitats. Environ. Microbiol. 18, 4974–4989. https://doi.org/10.1111/1462-2920.13455

Masia, C., Jensen P.E., Petersen, I.L., Buldo, P., 2022. Design of a functional pea protein matrix

for fermented plant-based cheese. Foods 11, 1–13. https://doi.org/10.3390/foods11020178

McClements, D.., Grossmann, L., 2021. A brief review of the science behind the design of healthy and sustainable plant-based foods. npj Sci. Food 5, 17. https://doi.org/10.1038/s41538-021-00099-y

McClements, D.J., Grossmann, L., 2022. Next-generation plant-based foods: design, production, and properties, Next-Generation Plant-based Foods. Springer International Publishing. https://doi.org/10.1007/978-3-030-96764-2

McSweeney, P.L.H., 2017. Biochemistry of cheese ripening: introduction and overview, in: Cheese: Chemistry, Physics and Microbiology. Elsevier Ltd, pp. 379–387. https://doi.org/10.1016/B978-0-12-417012-4.00014-4

McSweeney, P.L.H., Fox, P.F., Ciocia, F., 2017. Metabolism of residual lactose and of lactate and citrate, in: Cheese: Chemistry, Physics and Microbiology. Elsevier Ltd, pp. 411–421. https://doi.org/10.1016/B978-0-12-417012-4.00016-8

McSweeney, P.L.H., Fox, P.F., O'mahony, J.A., 2020. Advanced Dairy Chemistry: Volume 2: Lipids, Fourth Edition, Fourth Edi. ed. Springer International Publishing. https://doi.org/10.1007/978-3-030-48686-0/COVER

McSweeney, P.L.H., Sousa, M.J., 2000. Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. Lait 80, 293–324. https://doi.org/10.1051/lait:2000127

Medic, J., Atkinson, C., Hurburgh, C.R., 2014. Current knowledge in soybean composition. J. Am. Oil Chem. Soc. 91, 363–384. https://doi.org/10.1007/s11746-013-2407-9

Mefleh, M., Pasqualone, A., Caponio, F., Faccia, M., 2021. Legumes as basic ingredients in the

production of dairy-free cheese alternatives: a review. J. Sci. Food Agric. 102, 8–18. https://doi.org/10.1002/jsfa.11502

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428. https://doi.org/10.1021/ac60147a030

Montville, R., Schaffner, D.W., 2004. Analysis of published sprout seed sanitization studies shows treatments are highly variable. J. Food Prot. 67, 758–765. https://doi.org/10.4315/0362-028X-67.4.758

Morelli, C.F., Calvio, C., Biagiotti, M., Speranza, G., 2014. pH-Dependent hydrolase, glutaminase, transpeptidase and autotranspeptidase activities of *Bacillus subtilis* γ-glutamyltransferase. FEBS J. 281, 232–245. https://doi.org/10.1111/febs.12591

Møretrø, T., Langsrud, S., 2017. Residential bacteria on surfaces in the food industry and their implications for food safety and qualityb. Compr. Rev. Food Sci. Food Saf. 16, 1022–1041. https://doi.org/10.1111/1541-4337.12283

Morris, C.J., Thompson, J.F., 1962. The Isolation and Characterization of γ-l-Glutamyl-l-Tyrosine and γ-l-Glutamyl-l-Phenylalanine from Soybeans. Biochemistry 1, 706–709. https://doi.org/10.1021/bi00910a026

Mulyowidarso, R.K., Fleet, G.H., Buckle, K.A., 1990. Association of bacteria with the fungal fermentation of soybean tempe. J. Appl. Bacteriol. 68, 43–47. https://doi.org/10.1111/j.1365-2672.1990.tb02546.x

Mulyowidarso, R.K., Fleet, G.H., Buckle, K.A., 1989. The microbial ecology of soybean soaking for tempe production. Int. J. Food Microbiol. 8, 35–46. https://doi.org/10.1016/0168-1605(89)90078-0

Nakamoto, M., Fujii, T., Matsutomo, T., Kodera, Y., 2018. Isolation and identification of three γ-glutamyl tripeptides and their putative production mechanism in aged garlic extract. J. Agric. Food Chem. 66, 2891–2899. https://doi.org/10.1021/acs.jafc.7b05480

Nandakumar, R., Yoshimune, K., Wakayama, M., Moriguchi, M., 2003. Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. J. Mol. Catal. B Enzym. 23, 87–100. https://doi.org/10.1016/S1381-1177(03)00075-4

Nelson, G., Hoon, M.A., Chandrashekar, J., Zhang, Y., Ryba, N.J.P., Zuker, C.S., 2001. Mammalian sweet taste receptors. Cell 106, 381–390. https://doi.org/10.1016/S0092-8674(01)00451-2

Nishimura, T., Kuroda, M., Concept, K., 2019. Koku in Food Science and Physiology, Koku in Food Science and Physiology. https://doi.org/10.1007/978-981-13-8453-0

Nkhata, S.G., Ayua, E., Kamau, E.H., Shingiro, J.B., 2018. Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzymes. Food Sci. Nutr. 6, 2446–2458. https://doi.org/10.1002/fsn3.846

Nout, M.J.R., Beernink, G., Bonants-van Laarhoven, T.M.G., 1987a. Growth of *Bacillus cereus* in soyabean tempeh. Int. J. Food Microbiol. 4, 293–301. https://doi.org/10.1016/0168-1605(87)90004-3

Nout, M.J.R., Bonants-Van Laarhoven, T.M.G., de Dreu, R., Gerats, I.A.G.M., 1985. The influence of some process variables and storage conditions on the quality and shelf-life of soybean tempeh. Antonie Van Leeuwenhoek 51, 532–534. https://doi.org/10.1007/BF00404518

Nout, M.J.R., de Dreu, M.A., Zuurbier, A.M., Bonants-van Laarhoven, T.M.G., 1987b. Ecology

of controlled soyabean acidification for tempe manufacture. Food Microbiol. 4, 165–172. https://doi.org/10.1016/0740-0020(87)90032-3

Nout, M.J.R., Notermans, S., Rombouts, F.M., 1988. Effect of environmental conditions during soya-bean fermentation on the growth of *Staphylococcus aureus* and production and thermal stability of enterotoxins A and B. Int. J. Food Microbiol. 7, 299–309. https://doi.org/10.1016/0168-1605(88)90056-6

Nout, M.J.R., Rombouts, F.M., 1990. Recent developments in tempe research. J. Appl. Bacteriol. 69, 609–633. https://doi.org/10.1111/j.1365-2672.1990.tb01555.x

Ohsu, T., Amino, Y., Nagasaki, H., Yamanaka, T., Takeshita, S., Hatanaka, T., Maruyama, Y., Miyamura, N., Eto, Y., 2010. Involvement of the calcium-sensing receptor in human taste perception. J. Biol. Chem. 285, 1016–1022. https://doi.org/10.1074/jbc.M109.029165

Orlowski, M., Meister, A., 1971. Partial reactions catalyzed by γ-glutamylcysteine synthetase and evidence for an activated glutamate intermediate. J. Biol. Chem. 246, 7095–7105. https://doi.org/10.1016/s0021-9258(19)45858-4

Parente, E., Cogan, T.M., Powell, I.B., 2017. Starter cultures: General aspects, in: Cheese: Chemistry, Physics and Microbiology. Elsevier Ltd, pp. 201–226. https://doi.org/10.1016/B978-0-12-417012-4.00008-9

Pel, H.J., De Winde, J.H., Archer, D.B., Dyer, P.S., Hofmann, G., Schaap, P.J., Turner, G., De Vries, R.P., Albang, R., Albermann, K., Andersen, M.R., Bendtsen, J.D., Benen, J.A.E., Van Den Berg, M., Breestraat, S., Caddick, M.X., Contreras, R., Cornell, M., Coutinho, P.M., Danchin, E.G.J., Debets, A.J.M., Dekker, P., Van Dijck, P.W.M., Van Dijk, A., Dijkhuizen, L., Driessen, A.J.M., D'Enfert, C., Geysens, S., Goosen, C., Groot, G.S.P., De Groot, P.W.J.,

Guillemette, T., Henrissat, B., Herweijer, M., Van Den Hombergh, J.P.T.W., Van Den Hondel, C.A.M.J.J., Van Der Heijden, R.T.J.M., Van Der Kaaij, R.M., Klis, F.M., Kools, H.J., Kubicek, C.P., Van Kuyk, P.A., Lauber, J., Lu, X., Van Der Maarel, M.J.E.C., Meulenberg, R., Menke, H., Mortimer, M.A., Nielsen, J., Oliver, S.G., Olsthoorn, M., Pal, K., Van Peij, N.N.M.E., Ram, A.F.J., Rinas, U., Roubos, J.A., Sagt, C.M.J., Schmoll, M., Sun, J., Ussery, D., Varga, J., Vervecken, W., Van De Vondervoort, P.J.J., Wedler, H., Wösten, H.A.B., Zeng, A.P., Van Ooyen, A.J.J., Visser, J., Stam, H., 2007. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat. Biotechnol. 25, 221–231. https://doi.org/10.1038/nbt1282

Pereyra Gonzales, A.S., Naranjo, G.B., Leiva, G.E., Malec, L.S., 2010. Maillard reaction kinetics in milk powder: Effect of water activity at mild temperatures. Int. Dairy J. 20, 40–45. https://doi.org/10.1016/j.idairyj.2009.07.007

Pophaly, Sarang D., Singh, R., Pophaly, Saurabh D., Kaushik, J.K., Tomar, S.K., 2012. Current status and emerging role of glutathione in food grade lactic acid bacteria. Microb. Cell Fact. 11, 1–14. https://doi.org/10.1186/1475-2859-11-114

Portnoi, P.A., Macdonald, A., 2009. Determination of the lactose and galactose content of cheese for use in the galactosaemia diet. J. Hum. Nutr. Diet. 22, 400–408. https://doi.org/10.1111/j.1365-277X.2009.00948.x

Poste, L.M., Mackie, D.A., Butler, G., Larmond, E., 1991. Laboratory methods for sensory analysis of food. Agriculture Canada, Ottawa - Ontario. https://doi.org/publications.gc.ca/pub?id=9.699476&sl=0

Pripp, A.H., Ardö, Y., 2007. Modelling relationship between angiotensin-(I)-converting enzyme

inhibition and the bitter taste of peptides. Food Chem. 102, 880–888. https://doi.org/10.1016/j.foodchem.2006.06.026

Pswarayi, F., Gänzle, M.G., 2019. Composition and origin of the fermentation microbiota of mahewu, a Zimbabwean fermented cereal beverage. Appl. Environ. Microbiol. 85, 1–15. https://doi.org/10.1128/aem.03130-18

Pua, A., Tang, V.C.Y., Goh, R.M.V., Sun, J., Lassabliere, B., Liu, S.Q., 2022. Ingredients, processing, and fermentation: addressing the organoleptic boundaries of plant-based dairy analogues. Foods 11, 875. https://doi.org/10.3390/foods11060875

Qiao, N., Wittouck, S., Mattarelli, P., Zheng, J., Lebeer, S., Felis, G.E., Gänzle, M.G., 2022. After the storm - Perspectives on the taxonomy of *Lactobacillaceae*. JDS Commun. 3, 222–227. https://doi.org/10.3168/jdsc.2021-0183

Quattrini, M., Liang, N., Fortina, M.G., Xiang, S., Curtis, J.M., Gänzle, M., 2019. Exploiting synergies of sourdough and antifungal organic acids to delay fungal spoilage of bread. Int. J. Food Microbiol. 308, 8–14. https://doi.org/10.1016/j.ijfoodmicro.2018.09.007

Reddy, N.R., Pierson, M.D., Sathe, S.K., Salunkhe, D.K., Beuchat, L.R., 1983. Legume-based fermented foods: Their preparation and nutritional auality. Crit. Rev. Food Sci. Nutr. 17, 335–370. https://doi.org/10.1080/10408398209527353

Robinson, R.J., Fraaije, B.A., Clark, I.M., Jackson, R.W., Hirsch, P.R., Mauchline, T.H., 2016. Endophytic bacterial community composition in wheat (*Triticum aestivum*) is determined by plant tissue type, developmental stage and soil nutrient availability. Plant Soil 405, 381–396. https://doi.org/10.1007/s11104-015-2495-4

Ross, P., Morgan, S., Hill, C., 2002. Preservation and fermentation: Past, present and future. Int.

J. Food Microbiol. 79, 3–16. https://doi.org/10.1016/S0168-1605(02)00174-5

Roudot-Algaron, F., Kerhoas, L., Le Bars, D., Einhorn, J., Gripon, J.C., 1994. Isolation of γ-glutamyl peptides from Comté cheese. J. Dairy Sci. 77, 1161–1166. https://doi.org/10.3168/jds.S0022-0302(94)77053-3

Ruiz Sella, S.R.B., Bueno, T., de Oliveira, A.A.B., Karp, S.G., Soccol, C.R., 2021. *Bacillus subtilis* natto as a potential probiotic in animal nutrition. Crit. Rev. Biotechnol. 41, 355–369. https://doi.org/10.1080/07388551.2020.1858019

Running, C.A., Craig, B.A., Mattes, R.D., 2015. Oleogustus: The unique taste of fat. Chem. Senses 40, 507–516. https://doi.org/10.1093/chemse/bjv036

Saito, T., Iso, N., Mizuno, H., Kaneda, H., Suyama, Y., Kawamura, S., Ōsawa, S., 1974. Conformational change of a natto mucin in solution. Agric. Biol. Chem. 38, 1941–1946. https://doi.org/10.1080/00021369.1974.10861435

Samson, R.A., Van Kooij, J.A., De Boer, E., 1987. Microbiological quality of commercial tempeh in the Netherlands. J. Food Prot. 50, 92–94. https://doi.org/10.4315/0362-028X-50.2.92

Sarwin, R., Walther, C., Laskawy, G., Butz, B., Grosch, W., 1992. Determination of free reduced and total glutathione in wheat flours by an isotope dilution assay. Z. Lebensm. Unters. Forsch. 195, 27–32. https://doi.org/10.1007/BF01197835

Savijoki, K., Ingmer, H., Varmanen, P., 2006. Proteolytic systems of lactic acid bacteria. Appl. Microbiol. Biotechnol. 71, 394–406. https://doi.org/10.1007/s00253-006-0427-1

Schmitt, N., Yu, G., Greve, R., McIntyre, L., 2018. Outbreak of *Salmonella* Weltevreden linked to fermented cashew nut cheese in Victoria, BC. Environ. Heal. Rev. 61, 74–81. https://doi.org/10.5864/d2018-017

Schwab, C., Mastrangelo, M., Corsetti, A., Gänzle, M., 2008. Formation of oligosaccharides and polysaccharides by *Lactobacillus reuteri* LTH5448 and *Weissella cibaria* 10M in sorghum sourdoughs. Cereal Chem. 85, 679–684. https://doi.org/10.1094/CCHEM-85-5-0679

Setlow, P., 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. 101, 514–525. https://doi.org/10.1111/j.1365-2672.2005.02736.x

Sforza, S., Galaverna, G., Schivazappa, C., Marchelli, R., Dossena, A., Virgili, R., 2006. Effect of extended aging of parma dry-cured ham on the content of oligopeptides and free amino acids. J. Agric. Food Chem. 54, 9422–9429. https://doi.org/10.1021/jf061312+

Shih, I.L., Chen, L.D., Wu, J.Y., 2010. Levan production using *Bacillus subtilis* natto cells immobilized on alginate. Carbohydr. Polym. 82, 111–117. https://doi.org/10.1016/j.carbpol.2010.04.030

Shih, I.L., Van, Y.T., 2001. The production of poly-(γ-glutamic acid) from microorganisms and its various applications. Bioresour. Technol. 79, 207–225. https://doi.org/10.1016/S0960-8524(01)00074-8

Shih, I.L., Yu, Y.T., Shieh, C.J., Hsieh, C.Y., 2005. Selective production and characterization of levan by *Bacillus subtilis* (Natto) Takahashi. J. Agric. Food Chem. 53, 8211–8215. https://doi.org/10.1021/jf058084o

Shutov, A.D., Vaintraub, I.A., 1987. Degradation of storage proteins in germinating seeds. Phytochemistry 26, 1557–1566. https://doi.org/10.1016/S0031-9422(00)82245-1

Signorini, C., Carpen, A., Coletto, L., Borgonovo, G., Galanti, E., Capraro, J., Magni, C., Abate, A., Johnson, S.K., Duranti, M., Scarafoni, A., 2018. Enhanced vitamin B12 production in an

innovative lupin tempeh is due to synergic effects of *Rhizopus* and *Propionibacterium* in cofermentation. Int. J. Food Sci. Nutr. 69, 451–457. https://doi.org/10.1080/09637486.2017.1386627

Sjamsuridzal, W., Khasanah, M., Febriani, R., Vebliza, Y., Oetari, A., Santoso, I., Gandjar, I., 2021. The effect of the use of commercial tempeh starter on the diversity of *Rhizopus* tempeh in Indonesia. Sci. Rep. 11, 1–10. https://doi.org/10.1038/s41598-021-03308-6

Song, Z., Hu, Y., Chen, X., Li, G., Zhong, Q., He, X., Xu, W., 2021. Correlation between bacterial community succession and propionic acid during gray sufu fermentation. Food Chem. 353, 129447. https://doi.org/10.1016/j.foodchem.2021.129447

Sooresh, M.M., Willing, B.P., Bourrie, B.C.T., 2023. Opportunities and challenges of understanding community assembly in spontaneous food fermentation. Foods 12, 1–16. https://doi.org/10.3390/FOODS12030673

Sparringa, R.A., Owens, J.D., 1999. Causes of alkalinization in tempe solid substrate fermentation. Enzyme Microb. Technol. 25, 677–681. https://doi.org/10.1016/S0141-0229(99)00097-6

Stark, T., Hofmann, T., 2005. Structures, sensory activity, and dose/response functions of 2,5-diketopiperazines in roasted cocoa nibs (*Theobroma cacao*). J. Agric. Food Chem. 53, 7222–7231. https://doi.org/10.1021/jf051313m

Stetina, M., Behr, J., Vogel, R.F., 2014. The transcriptional response of *Lactobacillus sanfranciscensis* DSM 20451T and its tcyB mutant lacking a functional cystine transporter to diamide stress. Appl. Environ. Microbiol. 80, 4114–4125. https://doi.org/10.1128/AEM.00367-14

Stipanuk, M.H., 2004. Sulfur amino acid metabolism: pathways for production and removal of

homocysteine and cysteine. Annu. Rev. Nutr. 24, 539–577. https://doi.org/10.1146/annurev.nutr.24.012003.132418

Strafella, S., Simpson, D.J., Khanghahi, M.Y., Angelis, M. De, Gänzle, M., Minervini, F., Crecchio, C., 2021. Comparative genomics and in vitro plant growth promotion and biocontrol traits of lactic acid bacteria from the wheat rhizosphere. Microorganisms 9, 1–18. https://doi.org/10.3390/MICROORGANISMS9010078

Sugahara, H., Kato, S., Nagayama, K., Sashihara, K., Nagatomi, Y., 2022. Heterofermentative lactic acid bacteria such as *Limosilactobacillus* as a strong inhibitor of aldehyde compounds in plant-based milk alternatives. Front. Sustain. Food Syst. 6, 1–10. https://doi.org/10.3389/FSUFS.2022.965986/BIBTEX

Sugawara, E., Ito, T., Odagiri, S., Kubota, K., Kobayashi, A., 1985. Comparison of compositions of odor components of natto and cooked soybeans. Agric. Biol. Chem. 49, 311–317. https://doi.org/10.1271/bbb1961.49.311

Suzuki, H., Yamada, C., Kato, K., 2007. γ-Glutamyl compounds and their enzymatic production using bacterial γ-glutamyltranspeptidase. Amino Acids 32, 333–340. https://doi.org/10.1007/s00726-006-0416-9

Świeca, M., Kordowska-Wiater, M., Pytka, M., Gawlik-Dziki, U., Bochnak, J., Złotek, U., Baraniak, B., 2018. *Lactobacillus plantarum* 299v improves the microbiological quality of legume sprouts and effectively survives in these carriers during cold storage and *in vitro* digestion. PLoS One 13, 1–13. https://doi.org/10.1371/journal.pone.0207793

Tachiki, T., Yamada, T., Mizuno, K., Ueda, M., Shiode, J.I., Fukami, H., 1998. γ-Glutamyl transfer reactions by glutaminase from *Pseudomonas nitroreducens* IFO 12694 and their application

for the syntheses of theanine and γ-glutamylmethylamide. Biosci. Biotechnol. Biochem. https://doi.org/10.1271/bbb.62.1279

Tachiki, T., Yamada, T., Ueda, M., Naemura, Y., Imamura, N., Hamada, Y.I., Shiode, J.I., 1996. Purification and some properties of glutaminase from *Pseudomonas nitroreducens* IFO 12694. Biosci. Biotechnol. Biochem. 60, 1160–1164. https://doi.org/10.1271/bbb.60.1160

Tan, G., Hu, M., Li, X., Pan, Z., Li, M., Li, L., Yang, M., 2020. High-throughput sequencing and metabolomics reveal differences in bacterial diversity and metabolites between red and white sufu. Front. Microbiol. 11, 1–12. https://doi.org/10.3389/fmicb.2020.00758

Tanaka, K., Komiyama, A., Sonomoto, K., Ishizaki, A., Hall, S., Stanbury, P., 2002. Two different pathways for D-xylose metabolism and the effect of xylose concentration on the yield coefficient of L-lactate in mixed-acid fermentation by the lactic acid bacterium Lactococcus lactis IO-1. Appl. Microbiol. Biotechnol. 60, 160–167. https://doi.org/10.1007/s00253-002-1078-5

Tanaka, T., Muramatsu, K., Kim, H., Watanabe, T., Takeyasu, M., Kanai, Y., Kiuchi, K., 1998. Comparison of volatile compounds from Chungkuk-Jang and Itohiki-Natto. Biosci Biotechnol Biochem 62, 1440–1444. https://doi.org/10.1271/bbb.62.1440

Tangyu, M., Muller, J., Bolten, C.J., Wittmann, C., 2019. Fermentation of plant-based milk alternatives for improved flavour and nutritional value. Appl. Microbiol. Biotechnol. 103, 9263–9275.

Taormina, P.J., Beuchat, L.R., Slutsker, L., 1999. Infections associated with eating seed sprouts: An international concern. Emerg. Infect. Dis. 5, 626–634. https://doi.org/10.3201/eid0505.990503

Teixeira, J.S., McNeill, V., Gänzle, M.G., 2012. Levansucrase and sucrose phoshorylase contribute to raffinose, stachyose, and verbascose metabolism by lactobacilli. Food Microbiol. 31, 278–284. https://doi.org/10.1016/j.fm.2012.03.003

Teixeira, J.S., Seeras, A., Sanchez-Maldonado, A.F., Zhang, C., Su, M.S.W., Gänzle, M.G., 2014. Glutamine, glutamate, and arginine- based acid resistance in *Lactobacillus reuteri*. Food Microbiol. 42, 172–180. https://doi.org/10.1016/j.fm.2014.03.015

Teng, D.F., Lin, C.S., Sieh, P.C., 2004. Fermented Tofu: Sufu and Stinky Tofu, in: Handbook of Food and Beverage Fermentation Technology. CRC Press, New York, pp. 656–670. https://doi.org/https://doi.org/10.1201/9780203913550

Thiele, C., Gänzle, M.G., Vogel, R.F., 2002. Contribution of sourdough lactobacilli, yeast, and cereal enzymes to the generation of amino acids in dough relevant for bread flavor. Cereal Chem. 79, 45–51. https://doi.org/10.1094/CCHEM.2002.79.1.45

Thierry, A., Collins, Y.F., Abeijón Mukdsi, M.C., McSweeney, P.L.H., Wilkinson, M.G., Spinnler, H.E., 2017. Lipolysis and metabolism of fatty acids in cheese, in: Cheese: Chemistry, Physics and Microbiology. Elsevier Ltd, pp. 423–444. https://doi.org/10.1016/B978-0-12-417012-4.00017-X

Thomas, T.D., Ellwood, D.C., Longyear, V.M.C., 1979. Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. J. Bacteriol. 138, 109–117. https://doi.org/10.1128/jb.138.1.109-117.1979

Thompson, F., Turner, H., Gering, R.K., Beau, K., 1964. γ-glutamyl transpeptidase in plants. Phytochemistry 3, 33–46. https://doi.org/https://doi.org/10.1016/S0031-9422(00)83992-8

Thulin, E., Linse, S., 1998. Expression and purification of human γ-glutamylcysteine synthetase.

Protein Expr. Purif. 13, 268–276. https://doi.org/10.1006/prep.1999.1028

Toelstede, S., Dunkel, A., Hofmann, T., 2009. A series of kokumi peptides impart the long-lasting mouthfulness of matured gouda cheese. J. Agric. Food Chem. 57, 1440–1448. https://doi.org/10.1021/jf803376d

Toelstede, S., Hofmann, T., 2009. Kokumi-active glutamyl peptides in cheeses and their biogeneration by *Penicillium roquefortii*. J. Agric. Food Chem. 57, 3738–3748. https://doi.org/10.1021/jf900280j

Toelstede, S., Hofmann, T., 2008. Quantitative studies and taste re-engineering experiments toward the decoding of the nonvolatile sensometabolome of Gouda cheese. J. Agric. Food Chem. 56, 5299–5307. https://doi.org/10.1021/jf800552n

Tomita, K., Yano, T., Kitagata, T., Kumagai, H., Tochikura, T., 1989. Formation of γ-glutamyl peptides by glutaminase of *Aspergillus oryzae*. Agric. Biol. Chem. 53, 1995–1996. https://doi.org/10.1080/00021369.1989.10869596

Tornadijo, M.E., Fresno, J.M., Bernardo, A., Martin Sarmiento, R., Carballo, J., 1995. Microbiological changes throughout the manufacturing and ripening of a Spanish goat's raw milk cheese (Armada variety). Lait 75, 551–570. https://doi.org/10.1016/0023-7302(96)83004-1

Tsai, Y.H., Chang, S.C., Kung, H.F., 2007. Histamine contents and histamine-forming bacteria in natto products in Taiwan. Food Control 18, 1026–1030. https://doi.org/10.1016/j.foodcont.2006.06.007

Tsuji, S., Tanaka, K., Takenaka, S., Yoshida, K. ichi, 2015. Enhanced secretion of natto phytase by *Bacillus subtilis*. Biosci. Biotechnol. Biochem. 79, 1906–1914.

https://doi.org/10.1080/09168451.2015.1046366

U.S. Food and Drug Administration, 1998. Guidance for industry: Guide to minimize microbial food safety hazards for fresh fruits and vegetables. Oral Microbiol. Immunol. 19, 210–213. https://doi.org/10.1111/j.0902-0055.2004.00136.x

Udomsil, N., Rodtong, S., Choi, Y.J., Hua, Y., Yongsawatdigul, J., 2011. Use of *Tetragenococcus halophilus* as a starter culture for flavor improvement in fish sauce fermentation. J. Agric. Food Chem. 59, 8401–8408. https://doi.org/10.1021/JF201953V

Ueda, Y., Sakaguchi, M., Hirayama, K., Miyajima, R., Kimizuka, A., 1990. Characteristic Flavor Constituents in Water Extract of Garlic. Agric. Biol. Chem. 54, 163–169. https://doi.org/10.1271/bbb1961.54.163

Ueda, Y., Tsubuku, T., Miyajima, R., 1994. Composition of sulfur-containing components in onion and their flavor characters. Biosci. Biotechnol. Biochem. 58, 108–110. https://doi.org/10.1271/bbb.58.108

Ueda, Y., Yonemitsu, M., Tsubuku, T., Sakaguchi, M., Miyajima, R., 1997. Flavor characteristics of glutathione in raw and cooked foodstuffs. Biosci. Biotechnol. Biochem. 61, 1977–1980. https://doi.org/10.1271/bbb.61.1977

van de Guchte, M., Penaud, S., Grimaldi, C., Barbe, V., Bryson, K., Nicolas, P., Robert, C., Oztas, S., Mangenot, S., Couloux, A., Loux, V., Dervyn, R., Bossy, R., Bolotin, A., Batto, J.M., Walunas, T., Gibrat, J.F., Bessières, P., Weissenbach, J., Ehrlich, S.D., Maguin, E., 2006. The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. PNAS 103, 9274–9279. https://doi.org/10.1073/pnas.0603024103

Van der Meulen, R., Scheirlinck, I., Van Schoor, A., Huys, G., Vancanneyt, M., Vandamme, P.,

De Vuyst, L., 2007. Population dynamics and metabolite target analysis of lactic acid bacteria during laboratory fermentations of wheat and spelt sourdoughs. Appl. Environ. Microbiol. 73, 4741–4750. https://doi.org/10.1128/AEM.00315-07

Vergauwen, B., De Vos, D., Van Beeumen, J.J., 2006. Characterization of the bifunctional γ-glutamate-cysteine ligase/glutathione synthetase (GshF) of *Pasteurella multocida*. J. Biol. Chem. 281, 4380–4394. https://doi.org/10.1074/jbc.M509517200

Vermeulen, N., Czerny, M., Gänzle, M.G., Schieberle, P., Vogel, R.F., 2007. Reduction of (E)-2-nonenal and (E,E)-2,4-decadienal during sourdough fermentation. J. Cereal Sci. 45, 78–87. https://doi.org/10.1016/j.jcs.2006.07.002

Vermeulen, N., Kretzer, J., Machalitza, H., Vogel, R.F., Gänzle, M.G., 2006. Influence of redox-reactions catalysed by homo- and hetero-fermentative lactobacilli on gluten in wheat sourdoughs. J. Cereal Sci. 43, 137–143. https://doi.org/10.1016/j.jcs.2005.08.006

Vermeulen, N., Pavlovic, M., Ehrmann, M.A., Gänzle, M.G., Vogel, R.F., 2005. Functional characterization of the proteolytic system of *Lactobacillus sanfranciscensis* DSM 20451$^{T}$during growth in sourdough. Appl. Environ. Microbiol. 71, 6260–6266. https://doi.org/10.1128/AEM.71.10.6260-6266.2005

Wan, H., Liu, T., Su, C., Ji, X., Wang, L., Zhao, Y., Wang, Z., 2020. Evaluation of bacterial and fungal communities during the fermentation of Baixi sufu, a traditional spicy fermented bean curd. J. Sci. Food Agric. 100, 1448–1457. https://doi.org/10.1002/jsfa.10151

Wang, L.J., Cheng, Y.Q., Yin, L.J., Bhandari, B., Saito, M., Li, L.T., 2010a. Changes during processing and sodium chloride supplementation on the physical and chemical properties of douchi. Int. J. Food Prop. 13, 131–141. https://doi.org/10.1080/10942910802256149

Wang, L.J., Mu, H.L., Liu, H.J., Bhandari, B., Saito, M., Li, L.T., 2010b. Volatile components in three commercial douchies, a chinese traditional salt-fermented soybean food. Int. J. Food Prop. 13, 1117–1133. https://doi.org/10.1080/10942910902968726

Wang, P., Ma, X., Wang, W., Xu, D., Zhang, X., Zhang, J., Sun, Y., 2019. Characterization of flavor fingerprinting of red sufu during fermentation and the comparison of volatiles of typical products. Food Sci. Hum. Wellness 8, 375–384. https://doi.org/10.1016/j.fshw.2019.11.004

Wang, S., Chang, Y., Liu, B., Chen, H., Sun, B., Zhang, N., 2021. Characterization of the key aroma-active compounds in yongchuan douchi (Fermented soybean) by application of the sensomics approach. Molecules 26, 3048. https://doi.org/10.3390/molecules26103048

Wang, Y., Gao, Y., Liang, W., Liu, Y., Gao, H., 2020. Identification and analysis of the flavor characteristics of unfermented stinky tofu brine during fermentation using SPME-GC–MS, e-nose, and sensory evaluation. J. Food Meas. Charact. 14, 597–612. https://doi.org/10.1007/s11694-019-00351-w

Wei, Q., Wolf-Hall, C.E., Chang, S.K.C., 2001. Natto characteristics as affected by steaming time, *Bacillus* strain, and fermentation time. J. Food Sci. 66, 167–173. https://doi.org/10.1111/j.1365-2621.2001.tb15601.x

Wels, M., Siezen, R., Van Hijum, S., Kelly, W.J., Bachmann, H., 2019. Comparative genome analysis of *Lactococcus lactis* indicates niche adaptation and resolves genotype/phenotype disparity. Front. Microbiol. 10, 4. https://doi.org/10.3389/fmicb.2019.00004

White, C.C., Viernes, H., Krejsa, C.M., Botta, D., Kavanagh, T.J., 2003. Fluorescence-based microtiter plate assay for glutamate-cysteine ligase activity. Anal. Biochem. 318, 175–180.

https://doi.org/10.1016/S0003-2697(03)00143-X

Wiesel, I., Rehm, H.J., Bisping, B., 1997. Improvement of tempe fermentations by application of mixed cultures consisting of *Rhizopus* sp. and bacterial strains. Appl. Microbiol. Biotechnol. 47, 218–225. https://doi.org/10.1007/s002530050916

Wolf, W.J., 1970. Soybean proteins: Their functional, chemical, and physical properties. J. Agric. Food Chem. 18, 969–976. https://doi.org/10.1021/jf60172a025

Wolfe, B.E., Button, J.E., Santarelli, M., Dutton, R.J., 2014. Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. Cell 158, 422–433. https://doi.org/10.1016/j.cell.2014.05.041

Won, S., Curtis, J., Gänzle, M., 2021. LC-MS/MS quantitation of α-amylase/trypsin inhibitor CM3 and glutathione during wheat sourdough breadmaking. J. Appl. Microbiol. https://doi.org/10.1111/JAM.15346

Wuyts, S., Van Beeck, W., Oerlemans, E.F.M., Wittouck, S., Claes, I.J.J., De Boeck, I., Weckx, S., Lievens, B., De Vuyst, L., Lebeer, S., 2018. Carrot juice fermentations as man-made microbial ecosystems dominated by lactic acid bacteria. Appl. Environ. Microbiol. 84, 00134–18. https://doi.org/10.1128/AEM.00134-18

Xie, C., Zeng, H., Li, J., Qin, L., 2018. Comprehensive explorations of nutritional, functional and potential tasty components of various types of sufu, a Chinese fermented soybean appetizer. Food Sci. Technol. 39, 105–114. https://doi.org/10.1590/fst.37917

Xu, D., Wang, P., Zhang, X., Zhang, J., Sun, Y., Gao, L., Wang, W., 2020. High-throughput sequencing approach to characterize dynamic changes of the fungal and bacterial communities during the production of sufu, a traditional Chinese fermented soybean food.

Food Microbiol. 86, 103340. https://doi.org/10.1016/j.fm.2019.103340

Yan, B., Chen, Y.Y., Wang, W., Zhao, J., Chen, W., Gänzle, M., 2018. γ-Glutamyl cysteine ligase of *Lactobacillus reuteri* synthesizes γ-glutamyl dipeptides in sourdough. J. Agric. Food Chem. 66, 12368–12375. https://doi.org/10.1021/acs.jafc.8b05056

Yan, J., Tong, H., 2023. An overview of bitter compounds in foodstuffs: Classifications, evaluation methods for sensory contribution, separation and identification techniques, and mechanism of bitter taste transduction. Compr. Rev. Food Sci. Food Saf. 22, 187–232. https://doi.org/10.1111/1541-4337.13067

Yan, Y., Wolkers-Rooijackers, J., Nout, M.J.R., Han, B., 2013. Microbial diversity and dynamics of microbial communities during back-slop soaking of soybeans as determined by PCR-DGGE and molecular cloning. World J. Microbiol. Biotechnol. 29, 1969–1974. https://doi.org/10.1007/s11274-013-1349-6

Yang, H., Yang, L., Zhang, J., Li, H., Tu, Z., Wang, X., 2019. Exploring functional core bacteria in fermentation of a traditional Chinese food, *Aspergillus*-type douchi. PLoS One 14, 1–14. https://doi.org/10.1371/journal.pone.0226965

Yang, J., Bai, W., Zeng, X., Cui, C., 2019. Gamma glutamyl peptides: the food source, enzymatic synthesis, kokumi-active and the potential functional properties – A review. Trends Food Sci. Technol. 91, 339–346. https://doi.org/10.1016/j.tifs.2019.07.022

Yang, J., Sun-Waterhouse, D., Cui, C., Dong, K., Wang, W., 2017. Synthesis and sensory characteristics of kokumi γ-[Glu]n-Phe in the presence of glutamine and phenylalanine: glutaminase from *Bacillus amyloliquefaciens* or *Aspergillus oryzae* as the catalyst. J. Agric. Food Chem. 65, 8696–8703. https://doi.org/10.1021/acs.jafc.7b03419

Yang, L., Yang, H.L., Tu, Z.C., Wang, X.L., 2016. High-throughput sequencing of microbial community diversity and dynamics during douchi fermentation. PLoS One 11, 1–19. https://doi.org/10.1371/journal.pone.0168166

Yang, Y., Meier, F., Lo, J.A., Yuan, W., Lee, V., Sze, P., Chung, H.J., Yuk, H.G., 2013. Overview of recent events in the microbiological safety of sprouts and new intervention technologies. Compr. Rev. Food Sci. Food Saf. 12, 265–280. https://doi.org/10.1111/1541-4337.12010

Yao, D., Xu, L., Wu, M., Wang, X., Zhu, L., Wang, C., 2021. Effects of microbial community succession on flavor compounds and physicochemical properties during CS sufu fermentation. LWT 152, 112313. https://doi.org/10.1016/j.lwt.2021.112313

Yoneya, T., 2004. Fermented soy products: tempeh, nattos, miso, and soy sauce. Handb. Veg. Preserv. Process. 252–272.

Yoshikawa, Y., Chen, P., Zhang, B., Scaboo, A., Orazaly, M., 2014. Evaluation of seed chemical quality traits and sensory properties of natto soybean. Food Chem. 153, 186–192. https://doi.org/10.1016/j.foodchem.2013.12.027

Yu, S., Liu, L., Bu, T., Zheng, J., Wang, W., Wu, J., Liu, D., 2022. Purification and characterization of hypoglycemic peptides from traditional Chinese soy-fermented douchi . Food Funct. 13, 3343–3352. https://doi.org/10.1039/d1fo03941e

Yvon, M., Rijnen, L., 2001. Cheese flavour formation by amino acid catabolism. Biotechnol. Adv. 11, 185–201. https://doi.org/10.1016/j.biotechadv.2005.11.005

Zhang, C., Brandt, M.J., Schwab, C., Gänzle, M., 2010. Propionic acid production by cofermentation of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough. Food Microbiol. 27, 390–395. https://doi.org/10.1016/j.fm.2009.11.019

Zhang, G., Xu, Z., Gao, Y., Huang, X., Zou, Y., Yang, T., 2015. Effects of germination on the nutritional properties, phenolic profiles, and antioxidant activities of buckwheat. J. Food Sci. 80, H1111–H1119. https://doi.org/10.1111/1750-3841.12830

Zhang, H., Kodera, T., Eto, Y., Mine, Y., 2016. γ-Glutamyl valine supplementation-induced mitigation of gut inflammation in a porcine model of colitis. J. Funct. Foods 24, 558–567. https://doi.org/10.1016/j.jff.2016.04.038

Zhang, H., Kovacs-Nolan, J., Kodera, T., Eto, Y., Mine, Y., 2015. γ-Glutamyl cysteine and γ-glutamyl valine inhibit TNF-α signaling in intestinal epithelial cells and reduce inflammation in a mouse model of colitis via allosteric activation of the calcium-sensing receptor. Biochim. Biophys. Acta 1852, 792–804. https://doi.org/10.1016/j.bbadis.2014.12.023

Zhang, J., Du, G.C., Zhang, Y., Liao, X.Y., Wang, M., Li, Y., Chen, J., 2010. Glutathione protects *Lactobacillus sanfranciscensis* against freeze-thawing, freeze-drying, and cold treatment. Appl. Environ. Microbiol. 76, 2989–2996. https://doi.org/10.1128/AEM.00026-09

Zhang, J.H., Tatsumi, E., Fan, J.F., Li, L.T., 2007. Chemical components of *Aspergillus*-type Douchi, a Chinese traditional fermented soybean product, change during the fermentation process. Int. J. Food Sci. Technol. 42, 263–268. https://doi.org/10.1111/j.1365-2621.2005.01150.x

Zhang, P., Li, H., Zhao, W., Xiong, K., Wen, H., Yang, H., Wang, X., 2022. Dynamic analysis of physicochemical characteristics and microbial communities of *Aspergillus*-type douchi during fermentation. Food Res. Int. 153, 110932. https://doi.org/10.1016/j.foodres.2021.110932

Zhang, W., Luo, Q., Zhu, Y., Ma, J., Cao, L., Yang, M., Wen, P., Zhang, Z., He, X., 2018.

Microbial diversity in two traditional bacterial douchi from Gansu province in northwest China using Illumina sequencing. PLoS One 13, 1–16. https://doi.org/10.1371/journal.pone.0194876

Zhang, Y., Zeng, T., Wang, H., Song, J., Suo, H., 2021. Correlation between the quality and microbial community of natural-type and artificial-type Yongchuan Douchi. LWT 140. https://doi.org/10.1016/j.lwt.2020.110788

Zhao, C.J., Gänzle, M.G., 2016. Synthesis of taste-active γ-glutamyl dipeptides during sourdough fermentation by *Lactobacillus reuteri*. J. Agric. Food Chem. 64, 7561–7568. https://doi.org/10.1021/acs.jafc.6b02298

Zhao, C.J., Hu, Y., Schieber, A., Gänzle, M., 2013. Fate of ACE-inhibitory peptides during the bread-making process: Quantification of peptides in sourdough, bread crumb, steamed bread and soda crackers. J. Cereal Sci. 57, 514–519. https://doi.org/10.1016/j.jcs.2013.02.009

Zhao, C.J., Kinner, M., Wismer, W., Gänzle, M.G., 2015. Effect of glutamate accumulation during sourdough fermentation with *Lactobacillus reuteri* on the taste of bread and sodium-reduced bread. Cereal Chem. 92, 224–230. https://doi.org/10.1094/CCHEM-07-14-0149-R

Zhao, C.J., Schieber, A., Gänzle, M.G., 2016. Formation of taste-active amino acids, amino acid derivatives and peptides in food fermentations – A review. Food Res. Int. 89, 39–47. https://doi.org/10.1016/j.foodres.2016.08.042

Zheng, J., Ruan, L., Sun, M., Gänzle, M., 2015a. A genomic view of lactobacilli and pediococci demonstrates that phylogeny matches ecology and physiology. Appl. Environ. Microbiol. 81, 7233–7243. https://doi.org/10.1128/AEM.02116-15

Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., O'toole, P.W.,

Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G., Lebeer, S., 2020. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus beijerinck* 1901, and union of Lactobacillaceae and Leuconostocaceae. Int. J. Syst. Evol. Microbiol. 70, 2782–2858. https://doi.org/10.1099/ijsem.0.004107

Zheng, J., Zhao, X., Lin, X.B., Gänzle, M., 2015b. Comparative genomics *Lactobacillus reuteri* from sourdough reveals adaptation of an intestinal symbiont to food fermentations. Sci. Rep. 5, 1–11. https://doi.org/10.1038/srep18234

Zheng, L., Regenstein, J.M., Teng, F., Li, Y., 2020. Tofu products : A review of their raw materials , processing conditions , and packaging. Compr. Rev. Food Sci. Food Saf. 19, 3683–3714. https://doi.org/10.1111/1541-4337.12640

# APPENDIX

**Table S5.1.** pH values of soy and lupin cheese during ripening for 14, 45, and 90 days. Plant cheeses were produced from ungerminated seeds, or from seeds germinated: LAB only, or LAB with *B. velezensis* or with *B. amyloliquefaciens*.

| Samples | pH value | | |
|---|---|---|---|
| | **14 d** | **45 d** | **90 d** |
| Soy cheese | | | |
| ungerminated | 3.66 ± 0.03 | 3.67 ± 0.05 | 3.70 ± 0.02 |
| germinated with LAB | 3.79 ± 0.07 | 3.98 ± 0.13 | 3.98 ± 0.22 |
| germinated with LAB and *B. velezensis* | 3.99 ± 0.04 | 4.09 ± 0.09 | 4.12 ± 0.05 |
| germinated with LAB and *B. amyloliquefaciens* | 4.25 ± 0.06 | 4.20 ± 0.06 | 4.20 ± 0.04 |
| Lupine cheese | | | |
| ungerminated | 3.70 ± 0.08 | 3.78 ± 0.10 | 3.83 ± 0.08 |
| germinated with LAB | 3.80 ± 0.03 | 3.91 ± 0.02 | 3.98 ± 0.03 |
| germinated with LAB and *B. velezensis* | 4.07 ± 0.12 | 4.14 ± 0.15 | 4.15 ± 0.13 |

**Table S5.2.** Concentration of organic acids in soy cheese during ripening for 14 and 90 days. Plant cheeses were produced from ungerminated seeds, or from seeds germinated: LAB only, or LAB with *B. velezensis* or with *B. amyloliquefaciens*.

| Samples | Conc. (µmol/g dry weight) | |
|---|---|---|
| | **lactate** | **acetate** |
| 14 d | | |
| ungerminated | 377.8 ± 23.3 | 47.2 ± 9.4 |
| germinated with LAB | 154.9 ± 11.8 | 61.7 ± 33.3 |
| germinated with LAB and *B. velezensis* | 112.3 ± 14.8 | 76.8 ± 4.7 |
| germinated with LAB and *B. amyloliquefaciens* | 12.6 ± 2.8 | 140.6 ± 14.0 |
| 90 d | | |
| ungerminated | 384.2 ± 58.5 | 76.8 ± 5.0 |
| germinated with LAB | 89.4 ± 0.9 | 109.5 ± 19.1 |
| germinated with LAB and *B. velezensis* | 10.7 ± 5.3 | 153.7 ± 12.8 |

**Table S6.1.** Relative abundance (%) of starter/adjunct cultures in soy cheese ripened for 0, 14, 45, and 90 days as determined by high throughput sequencing of full length 16S rRNA genes.

| Samples | | *Lc. lactis* | *Lp. plantarum* | Adjunct culture[a)] | *Bacillus* spp. | Total | # of reads / 1000 |
|---|---|---|---|---|---|---|---|
| | | | 0 d | | | | |
| ungerminated | *Lv. hammesii* | 33.3±2.6 | 63.4±2.4 | 2.7±0.4 | 0 | 99.3±0.2 | 0.8—20.1 |
| | *Ff. milii* | 38.7±3.3 | 52.7±4.7 | 7.9±1.7 | 0 | 99.2±0.3 | 7.3—11.6 |
| | *Lt. buchneri* | 43.7±2.4 | 54.0±1.5 | 1.0±0.5 | 0 | 98.7±0.7 | 7.9—15.9 |
| Germinated with LAB | *Lv. hammesii* | 45.7±13.6 | 51.4±12.9 | 2.3±0.8 | 0 | 99.4±0.2 | 0.6—5.4 |
| | *Ff. milii* | 46.0±9.6 | 44.3±9.2 | 8.9±2.0 | 0 | 99.2±0.4 | 12.1—16.7 |
| | *Lt. buchneri* | 63.1±13.0 | 34.8±12.9 | 1.4±0.9 | 0 | 99.3±0.3 | 17.0—28.7 |
| Germinated with LAB and *B. velezensis* | *Lv. hammesii* | 40.8±17.9 | 54.7±17.6 | 3.2±0.5 | 0 | 98.7±0.1 | 3.6—11.4 |
| | *Ff. milii* | 36.1±7.7 | 47.6±3.8 | 15.1±3.9 | 0 | 98.8±0.2 | 8.9—22.5 |
| | *Lt. buchneri* | 34.6±7.1 | 62.5±8.6 | 1.4±0.8 | 0 | 98.5±0.8 | 9.3—21.9 |
| Germinated with LAB and *B. amyloliquefaciens* | *Lv. hammesii* | 17.3±7.7 | 77.2±7.8 | 2.8±0.2 | 0.6±0.4 | 97.8±0.5 | 7.5—16.4 |
| | *Ff. milii* | 24.0±5.7 | 62.5±6.7 | 9.8±0.2 | 1.0±0.8 | 97.3±1.7 | 9.6—13.3 |
| | *Lt. buchneri* | 21.5±4.1 | 71.8±4.1 | 2.5±0.7 | 1.0±1.2 | 96.8±1.6 | 11.2—17.6 |
| | | | 14 d | | | | |
| ungerminated | *Lv. hammesii* | 35.5±6.1 | 59.1±5.0 | 4.1±0.3 | 0 | 98.6±1.1 | 0.3—6.1 |
| | *Ff. milii* | 42.5±1.9 | 48.4±2.0 | 8.5±0.6 | 0 | 99.4±0.1 | 1.8—4.7 |
| | *Lt. buchneri* | 43.7±6.4 | 55.2±6.0 | 0.4±0.2 | 0 | 99.3±0.3 | 1.7—5.3 |
| Germinated with LAB | *Lv. hammesii* | 39.6±15.3 | 57.3±15.6 | 1.9±0.3 | 0 | 98.8±0.3 | 0.3—3.7 |
| | *Ff. milii* | 41.6±14.2 | 49.5±13.9 | 8.0±1.3 | 0 | 99.2±0.3 | 3.9—5.5 |
| | *Lt. buchneri* | 38.4±7.1 | 59.0±6.7 | 1.8±0.4 | 0 | 99.2±0.2 | 0.5—5.2 |
| Germinated with LAB and *B. velezensis* | *Lv. hammesii* | 38.5±19.2 | 57.3±18.6 | 2.9±0.8 | 0 | 98.7±0.2 | 7.6—9.2 |
| | *Ff. milii* | 35.9±7.3 | 47.1±2.6 | 16.5±4.9 | 0 | 99.5±0.4 | 0.4—5.3 |
| | *Lt. buchneri* | 32.2±1.7 | 65.5±2.4 | 1.0±0.1 | 0 | 98.7±0.6 | 3.6—13.1 |
| Germinated with LAB and *B. amyloliquefaciens* | *Lv. hammesii* | 26.3±15.9 | 68.7±15.7 | 3.4±0.6 | 0.3±0.3 | 98.6±0.2 | 4.4—14.4 |
| | *Ff. milii* | 22.4±7.6 | 64.4±6.4 | 11.3±1.8 | 0.2±0.3 | 98.4±1.1 | 3.0—12.9 |
| | *Lt. buchneri* | 22.9±2.5 | 70.9±3.8 | 2.3±0.8 | 0.4±0.4 | 96.4±3.4 | 1.5—6.4 |

| Samples | | *Lc. lactis* | *Lp. plantarum* | Adjunct culture[a)] | *Bacillus* spp. | Total | # of reads / 1000 |
|---|---|---|---|---|---|---|---|
| | | | 45 d | | | | |
| ungerminated | *Lv. hammesii* | 35.4±6.0 | 58.5±5.2 | 5.3±1.0 | 0 | 99.2±0.4 | 14.5—19.6 |
| | *Ff. milii* | 43.2±4.7 | 42.9±5.3 | 13.5±0.8 | 0 | 99.5±0.2 | 1.8—16.1 |
| | *Lt. buchneri* | 49.6±5.9 | 47.8±6.3 | 1.8±0.4 | 0 | 99.3±0.1 | 0.3—5.6 |
| Germinated with LAB | *Lv. hammesii* | 36.0±13.4 | 60.3±13.0 | 3.0±0.9 | 0 | 99.4±0.2 | 5.7—7.8 |
| | *Ff. milii* | 26.8±15.8 | 64.5±14.1 | 7.8±1.8 | 0 | 99.2±0.1 | 5.0—13.7 |
| | *Lt. buchneri* | 30.2±7.3 | 67.2±6.6 | 1.5±0.5 | 0 | 99.0±0.3 | 9.4—11.4 |
| Germinated with LAB and *B. velezensis* | *Lv. hammesii* | 33.7±14.5 | 61.5±13.9 | 3.8±1.0 | 0 | 99.0±0.3 | 7.5—18.4 |
| | *Ff. milii* | 38.0±12.1 | 46.1±7.0 | 15.3±5.6 | 0 | 99.4±0.1 | 2.4—17.8 |
| | *Lt. buchneri* | 36.4±0.7 | 60.8±1.4 | 1.8±0.4 | 0 | 99.0±0.5 | 18.6—32.5 |
| Germinated with LAB and *B. amyloliquefaciens* | *Lv. hammesii* | 15.1±6.9 | 79.9±7.0 | 3.6±0.5 | 0.2±0.2 | 98.7±0.3 | 10.2—22.0 |
| | *Ff. milii* | 18.6±2.9 | 71.6±2.7 | 8.4±0.9 | 0.5±0.6 | 99.1±0.5 | 7.2—32.1 |
| | *Lt. buchneri* | 20.4±5.5 | 74.4±7.1 | 2.6±0.6 | 0.6±0.6 | 98.0±1.4 | 6.1—14.4 |
| | | | 90 d | | | | |
| ungerminated | *Lv. hammesii* | 52.4±6.7 | 42.4±5.7 | 4.5±0.8 | 0 | 99.2±0.4 | 4.3—13.5 |
| | *Ff. milii* | 54.4±3.7 | 22.8±6.2 | 22.3±2.6 | 0 | 99.5±0.1 | 9.0—14.2 |
| | *Lt. buchneri* | 56.8±9.2 | 40.0±8.5 | 2.5±0.6 | 0 | 99.3±0.2 | 9.1—10.1 |
| Germinated with LAB | *Lv. hammesii* | 29.3±6.5 | 67.4±8.0 | 2.2±0.4 | 0 | 98.9±1.4 | 1.0—11.7 |
| | *Ff. milii* | 30.3±9.7 | 62.1±10.6 | 7.1±1.4 | 0 | 99.5±0.2 | 1.8—5.8 |
| | *Lt. buchneri* | 27.0±8.5 | 71.2±7.7 | 1.3±0.8 | 0 | 99.5±0.1 | 1.3—61.6 |
| Germinated with LAB and *B. velezensis* | *Lv. hammesii* | 44.6±12.8 | 50.6±11.8 | 4.1±1.7 | 0 | 99.3±0.3 | 0.4—6.4 |
| | *Ff. milii* | 38.2±12.0 | 43.8±9.1 | 17.3±4.2 | 0 | 99.3±0.4 | 0.7—5.3 |
| | *Lt. buchneri* | 37.3±5.3 | 59.0±4.2 | 2.7±1.1 | 0 | 99.0±0.2 | 7.4—15.4 |
| Germinated with LAB and *B. amyloliquefaciens* | *Lv. hammesii* | 19.9±7.7 | 75.3±8.6 | 3.2±1.0 | 0.3±0.3 | 98.7±0.5 | 9.6—22.6 |
| | *Ff. milii* | 21.1±9.5 | 67.4±9.7 | 10.4±1.3 | 0.2±0.1 | 99.2±0.2 | 4.2—9.4 |
| | *Lt. buchneri* | 20.5±1.3 | 74.7±2.2 | 2.6±0.6 | 0.5±0.6 | 98.3±0.7 | 0.3—10.6 |

[a)] lactobacilli species inoculated as an adjunct;

**Table S6.2.** Relative abundance (%) of starter cultures in lupine cheese ripened for 0, 14, 45, and 90 days as determined by high throughput sequencing of full length 16S rRNA genes.

| Samples | | *Lc. lactis* | *Lp. plantarum* | Adjunct culture[a)] | *Bacillus* spp. | Total | # of reads / 1000 |
|---|---|---|---|---|---|---|---|
| | | | 0 d | | | | |
| ungerminated | *Lv. hammesii* | 9.7± 3.0 | 86.1±2.3 | 3.6±0.8 | 0 | 99.4±0.0 | 7.3—22.5 |
| | *Ff. milii* | 9.0±1.7 | 81.5±2.6 | 9.0±2.2 | 0 | 99.5±0.2 | 1.8—21.5 |
| | *Lt. buchneri* | 10.4±0.4 | 85.5±0.6 | 3.5±0.2 | 0 | 99.4±0.1 | 6.1—9.6 |
| Germinated with LAB | *Lv. hammesii* | 11.4±1.3 | 83.3±1.1 | 4.6±0.0 | 0 | 99.3±0.2 | 2.3—3.3 |
| | *Ff. milii* | 6.2±6.1 | 66.2±13.3 | 27.0±9.1 | 0 | 99.4±0.2 | 0.9—21.2 |
| | *Lt. buchneri* | 3.1±2.4 | 88.2±5.1 | 7.6±2.8 | 0 | 98.9±0.1 | 2.9—4.9 |
| Germinated with LAB and *B. velezensis* | *Lv. hammesii* | 37.3±114 | 48.1±8.0 | 12.5±6.6 | 0 | 98.0±2.5 | 6.1—17.8 |
| | *Ff. milii* | 20.8±6.8 | 38.0±14.7 | 39.8±9.8 | 0 | 98.5±1.9 | 5.9—18.5 |
| | *Lt. buchneri* | 27.4±16.8 | 59.3±17.3 | 11.3±7.2 | 0 | 97.9±2.2 | 1.0—30.4 |
| | | | 14 d | | | | |
| ungerminated | *Lv. hammesii* | 8.5±2.5 | 86.9±2.5 | 4.0±0.5 | 0 | 99.5±0.1 | 7.0—15.4 |
| | *Ff. milii* | 6.8±3.4 | 85.4±3.9 | 7.4±0.8 | 0 | 99.5±0.1 | 1.4—5.4 |
| | *Lt. buchneri* | 8.3±2.5 | 88.5±3.4 | 2.8±1.0 | 0 | 99.6±0.1 | 2.6—24.9 |
| Germinated with LAB | *Lv. hammesii* | 5.7±4.7 | 90.4±5.3 | 3.4±0.7 | 0 | 99.5±0.1 | 1.0—11.3 |
| | *Ff. milii* | 4.1±4.1 | 79.7±11.0 | 15.9±7.4 | 0 | 99.6±0.1 | 10.0—18.8 |
| | *Lt. buchneri* | 2.0±1.6 | 94.4±2.3 | 3.1±0.8 | 0 | 99.5±0.0 | 7.5—15.2 |
| Germinated with LAB and *B. velezensis* | *Lv. hammesii* | 40.8±18.6 | 49.2±19.7 | 8.5±2.8 | 0 | 98.5±1.6 | 0.2—20.5 |
| | *Ff. milii* | 21.2±13.7 | 41.0±20.2 | 37.2±27.8 | 0 | 99.4±0.7 | 5.1—15.2 |
| | *Lt. buchneri* | 32.1±19.9 | 60.1±21.3 | 6.6±2.3 | 0 | 98.8±1.1 | 3.4—13.0 |

| Samples | | *Lc. lactis* | *Lp. plantarum* | Adjunct culture[a)] | *Bacillus* spp. | Total | # of reads / 1000 |
|---|---|---|---|---|---|---|---|
| | | | 45 d | | | | |
| ungerminated | *Lv. hammesii* | 11.2±0.8 | 80.5±3.6 | 7.3±3.0 | 0 | 98.9±1.3 | 2.6—4.8 |
| | *Ff. milii* | 7.6±4.1 | 82.6±5.2 | 9.6±1.2 | 0 | 99.8±0.1 | 1.3—23.4 |
| | *Lt. buchneri* | 10.2±3.4 | 86.9±3.5 | 2.7±0.5 | 0 | 99.8±0.1 | 3.9—10.2 |
| Germinated with LAB | *Lv. hammesii* | 6.3±5.8 | 90.5±4.9 | 2.8±0.9 | 0 | 99.7±0.1 | 0.3—9.4 |
| | *Ff. milii* | 5.0±6.2 | 74.7±18.1 | 19.8±11.8 | 0 | 99.6±0.0 | 6.8—23.9 |
| | *Lt. buchneri* | 2.2±1.8 | 93.9±3.3 | 3.5±1.7 | 0 | 99.6±0.1 | 9.3—17.8 |
| Germinated with LAB and *B. velezensis* | *Lv. hammesii* | 49.4±17.1 | 42.8±16.3 | 7.4±2.9 | 0 | 99.6±0.4 | 23.9—45.6 |
| | *Ff. milii* | 22.1±16.7 | 32.0±14.6 | 45.7±25.8 | 0 | 99.8±0.2 | 0.8—12.7 |
| | *Lt. buchneri* | 31.3±12.6 | 60.1±15.3 | 8.3±2.7 | 0 | 99.6±0.0 | 2.8—10.1 |
| | | | 90 d | | | | |
| ungerminated | *Lv. hammesii* | 18.0±2.7 | 75.8±2.5 | 5.5±0.4 | 0 | 99.3±0.2 | 1.3—12.3 |
| | *Ff. milii* | 11.0±4.8 | 68.0±5.5 | 20.6±4.9 | 0 | 99.6±0.1 | 8.3—18.3 |
| | *Lt. buchneri* | 13.1±2.9 | 83.5±4.1 | 2.9±1.2 | 0 | 99.5±0.1 | 5.7—25.5 |
| Germinated with LAB | *Lv. hammesii* | 10.6±10.2 | 84.8±11.8 | 4.1±1.7 | 0 | 99.4±0.1 | 1.6—35.8 |
| | *Ff. milii* | 5.3±5.5 | 74.6±15.4 | 19.5±10.4 | 0 | 99.5±0.2 | 3.9—50.5 |
| | *Lt. buchneri* | 2.2±1.7 | 94.0±2.8 | 3.3±1.1 | 0 | 99.5±0.1 | 1.8—14.7 |
| Germinated with LAB and *B. velezensis* | *Lv. hammesii* | 52.7±13.9 | 39.6±15.3 | 6.6±1.6 | 0 | 99.0±0.2 | 6.4—7.5 |
| | *Ff. milii* | 41.1±11.2 | 33.0 ±14.6 | 25.6±3.6 | 0 | 99.7±0.1 | 9.5—38.1 |
| | *Lt. buchneri* | 45.7±11.7 | 45.8±12.2 | 7.6±0.8 | 0 | 99.0±0.1 | 11.1—25.9 |

[a)] lactobacilli species inoculated as an adjunct;

**Table S6.3.** Concentrations of free amino nitrogen in soy cheeses with adjunct cultures ripened for 0, 14, 45, and 90 days.

| Samples | Conc. (µmol/g dry weight) | | | |
|---|---|---|---|---|
| | **0 d** | **14 d** | **45 d** | **90 d** |
| No adjunct culture | | | | |
| ungerminated | 67.9±2.8$^{c}$ | 100.6±5.5$^{c}$ | 181.3±18.7$^{c*}$ | 218.7±28.7$^{b}$ |
| Germinated with LAB | 102.6±10.9$^{bc}$ | 117.0±14.9$^{bc}$ | 157.6±8.2$^{bc*}$ | 218.1±62.5$^{b}$ |
| Germinated with LAB and *B. velezensis* | 175.5±26.9$^{a}$ | 212.8±17.8$^{a}$ | 302.6±9.5$^{a*}$ | 370.1±21.1$^{a}$ |
| Germinated with LAB and *B. amyloliquefaciens* | 139.8±28.9$^{ab}$ | 172.1±42.8$^{ab}$ | 233.8±52.1$^{ab}$ | 291.0±44.9$^{ab}$ |
| *Lv. hammesii* | | | | |
| ungerminated | 69.8±1.8$^{b}$ | 100.3±7.9$^{c}$ | 179.9±15.6$^{b*}$ | 215.5±6.7$^{b}$ |
| Germinated with LAB | 98.9±9.3$^{b}$ | 111.9±14.7$^{c}$ | 182.3±10.8$^{b*}$ | 214.3±8.2$^{b}$ |
| Germinated with LAB and *B. velezensis* | 174.5±4.3$^{a}$ | 206.8±2.0$^{a}$ | 306.0±48.3$^{a*}$ | 372.2±23.2$^{a}$ |
| Germinated with LAB and *B. amyloliquefaciens* | 142.8±29.8$^{a}$ | 156.2±25.4$^{b}$ | 237.6±35.7$^{ab*}$ | 307.7±72.5$^{ab}$ |
| *Ff. milii* | | | | |
| ungerminated | 79.7±27.9$^{c}$ | 108.3±12.5 | 176.5±6.3b$^{*}$ | 243.2±21.2$^{b}$ |
| Germinated with LAB | 104.1±8.6$^{bc}$ | 122.8±5.8 | 192.8±11.7b$^{*}$ | 283.4±24.0$^{b}$ |
| Germinated with LAB and *B. velezensis* | 168.2±11.5$^{a}$ | 224.0±20.1 | 330.7±13.9$^{a*}$ | 435.2±77.8$^{a}$ |
| Germinated with LAB and *B. amyloliquefaciens* | 128.6±18.1$^{ab}$ | 129.0±87.1 | 231.6±63.3$^{b}$ | 332.9±60.0$^{ab}$ |
| *Lt. buchneri* | | | | |
| ungerminated | 64.4±10.1$^{b}$ | 96.8±11.9$^{b}$ | 183.4±13.8$^{b*}$ | 220.9±35.2$^{c}$ |
| Germinated with LAB | 96.5±9.4$^{b}$ | 111.4±12.7$^{b}$ | 173.7±14.9$^{b*}$ | 243.3±9.9$^{bc}$ |
| Germinated with LAB and *B. velezensis* | 162.6±15.9$^{a}$ | 202.1±4.5$^{a}$ | 305.2±12.3$^{a*}$ | 363.7±20.7$^{a}$ |
| Germinated with LAB and *B. amyloliquefaciens* | 137.0±21.1$^{a}$ | 167.2±32.7$^{a}$ | 250.5±42.4$^{a*}$ | 326.5±59.7$^{ab}$ |

Values for each adjunct culture in the same column that do not share a lowercase superscript differ significantly ($P$<0.05). Significant differences ($P$<0.05) between unripened cheeses and cheeses ripened for 45 days are indicated with an asterisk.

**Table S6.4.** Concentrations of free amino nitrogen in lupine cheeses with adjunct cultures ripened for 0, 14, 45, and 90 days

| Samples | Conc. (µmol/g dry weight) | | | |
|---|---|---|---|---|
| | **0 d** | **14 d** | **45 d** | **90 d** |
| | No adjunct culture | | | |
| ungerminated | 124.5±11.7[b] | 211.5±8.1[b] | 321.3±5.2[*] | 359.1±17.5 |
| Germinated with LAB | 148.6±18.9[ab] | 262.2±26.7[b] | 366.4±75.4[*] | 363.3±50.7 |
| Germinated with LAB and *B. velezensis* | 171.8±16.9[a] | 282.1±17.2[a] | 436.5±69.8[*] | 413.5±65.6 |
| | *Lv. hammesii* | | | |
| ungerminated | 127.4±4.3 | 247.5±18.1[b] | 365.8±22.1[*] | 357.9±75.9 |
| Germinated with LAB | 144.4±29.8 | 250.8±12.2[b] | 447.6±36.4[*] | 403.4±29.9 |
| Germinated with LAB and *B. velezensis* | 175.5±39.2 | 285.8±9.5[a] | 483.9±80.5[*] | 386.4±40.7 |
| | *Ff. milii* | | | |
| ungerminated | 132.2±8.7 | 246.8±18.3 | 425.6±33.7[*] | 419.1±46.4 |
| Germinated with LAB | 141.5±22.7 | 269.5±10.3 | 453.5±66.1[*] | 471.0±39.5 |
| Germinated with LAB and *B. velezensis* | 170.7±20.1 | 288.3±25.4 | 502.1±103.3[*] | 462.1±81.3 |
| | *Lt. buchneri* | | | |
| ungerminated | 133.1±12.3 | 235.3±13.5 | 442.3±23.7[*] | 420.9±17.2 |
| Germinated with LAB | 143.5±21.7 | 306.2±43.0 | 493.2±129.5[*] | 459.6±28.0 |
| Germinated with LAB and *B. velezensis* | 167.3±9.2 | 301.3±60.5 | 427.4±83.3[*] | 462.9±83.8 |

Values for each adjunct culture in the same column that do not share a lowercase superscript differ significantly ($P<0.05$). Significant differences ($P<0.05$) between unripened cheeses and cheeses ripened for 45 days are indicated with an asterisk.

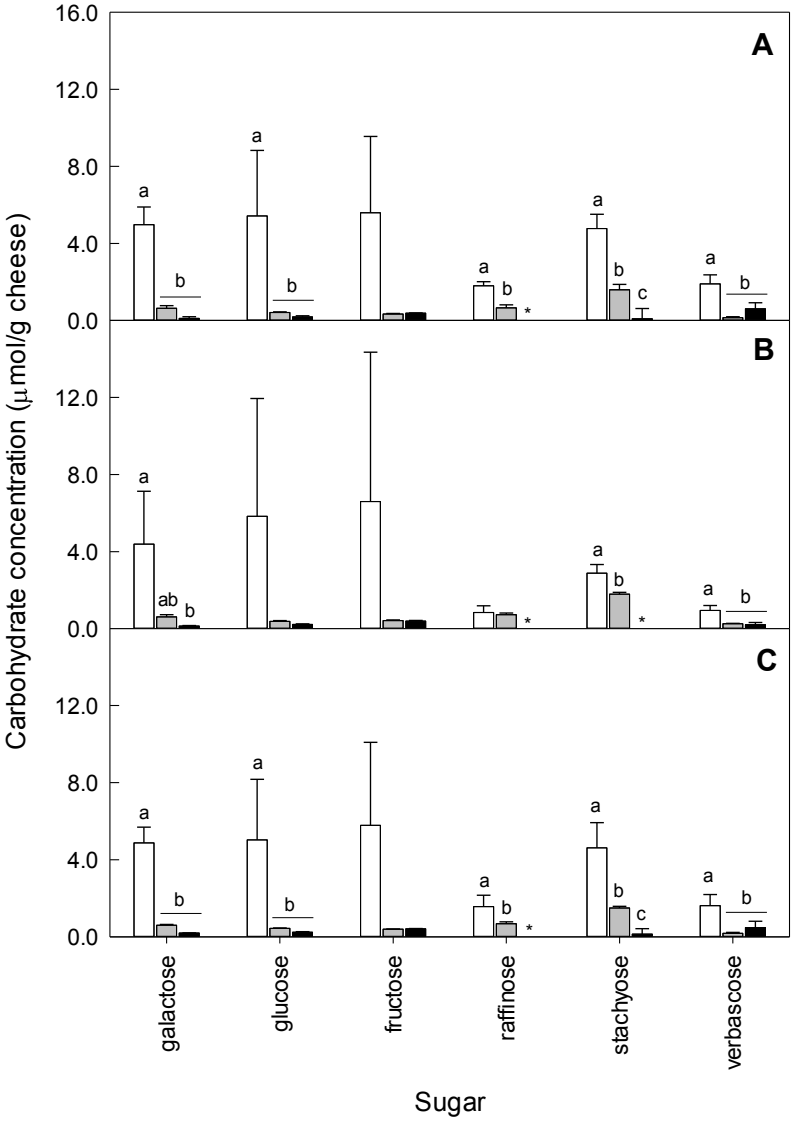



**Figure S6.1.** Carbohydrate concentrations in unripened soy cheeses inoculated with: *Lv. hammesii* (A), *Ff. milii* (B), and *Lt. buchneri* (C). Colours indicate carbohydrate concentrations in unripened soy cheeses made from ungerminated seeds (white) or seeds germinated: with LAB only (grey) or LAB with *B. velezensis* (black). "not detected" means the signal was lower than detection limits and indicated with an asterisk.

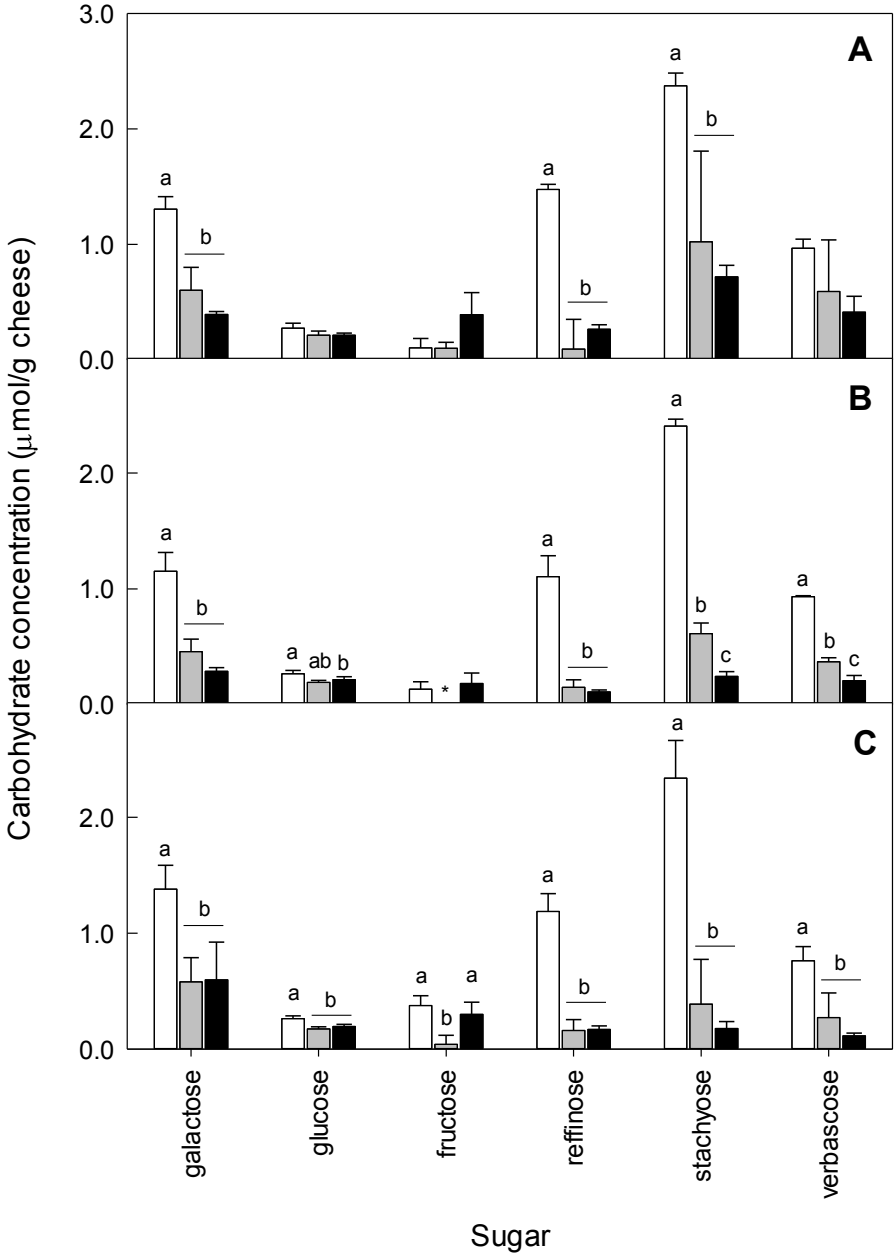



**Figure S6.2.** Carbohydrate concentrations in unripened lupine cheeses inoculated with: *Lv. hammesii* (A), *Ff. milii* (B), and *Lt. buchneri* (C). Colours indicate carbohydrate concentrations in unripened lupine cheeses made from ungerminated seeds (white) or seeds germinated: with LAB only (grey) or LAB with *B. velezensis* (black). "not detected" means the signal was lower than detection limits and indicated with an asterisk.