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Rye sourdough fermentation and bread stability

ANNA MIHHALEVSKI

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TALLINN UNIVERSITY OF TECHNOLOGY
Faculty of Chemical and Material Technology
Department of Food Processing

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Supervisor: Professor Toomas Paalme,
Chair of Food Processing, Department of Food Processing,
Tallinn University of Technology (TUT)

Opponents: Professor Hannu Salovaara,
Department of Food and Environmental Sciences,
University of Helsinki, Finland

Professor Rimantas Venskutonis,
Department of Food Technology,
Kaunas University of Technology, Lithuania

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DECLARATION: I hereby declare that this doctoral thesis, submitted for the doctoral degree at TUT, is my original investigation and achievement and has not been submitted for the defense of any academic degree elsewhere.

Anna Mihhalevski

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ANNA MIHHALEVSKI

TTÜ KIRJASTUS

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ABSTRACT

Although research on rye bread quality and processing has been carried out for over a century, a number of factors that determine both the stability of rye sourdough fermentation, and bread quality during storage are incompletely understood.

A descriptive sensory analysis comparing rye sourdough bread with wheat bread was carried out. The results show that rye sourdough bread retains its quality properties better during storage. This study provides insight into important aspects of sourdough bread processing relating to quality issues and staling.

A laboratory rye sourdough process that simulates industrial processing was developed and used to study the effect of individual processing parameters on the quality and stability of rye bread. The microbiological and chemical processes that occur during sourdough fermentation were studied using traditional techniques as well as isothermal microcalorimetry. The latter technique provides transient growth rate profiles of lactic acid bacteria (LAB) during sourdough fermentation and was applied, together with chromatographic methods, to compare the growth characteristics of commercial starter cultures and LAB isolated from both industrial and spontaneous laboratory sourdough. By observing heat production profiles and the transient formation of volatile compounds, sugars, organic acids, and free amino acids during fermentation, we conclude that acidity tolerance is a key factor affecting the formation of microbial consortia during repeated laboratory and industrial sourdough cycles. The essential carbon and nitrogen sources did not become depleted at any point during sourdough fermentation and the maximum specific growth rate of strains in sourdough was only a secondary growth limitation factor. In addition, it was observed that the strains isolated from industrial sourdough were sensitive to oxygen.

We also studied the effect of bread processing on the content of soluble and insoluble fiber, as well as vitamers in bread. The content of thiamine (98-140 $\mu g \cdot 100 g_{DM}^{-1}$), nicotinic acid (57-210 $\mu g \cdot 100 g_{DM}^{-1}$), pyridoxine (24-42 $\mu g \cdot 100 g_{DM}^{-1}$), and riboflavin (50-80 $\mu g \cdot 100 g_{DM}^{-1}$) in breads was found to be 40% lower than that in the raw materials. In contrast, the content of nicotinamide increased tenfold during processing, presumably due to microbial activity during sourdough fermentation. During rye sourdough processing, the ratio of soluble to insoluble dietary fiber increased, along with total dietary fiber. Those peculiarities of rye bread processing should be taken into account both in the nutritional labeling of commercial products and when calculating the functional compounds in bread based on the ingredients in the recipe.

Finally, we studied the stability of both rye sourdough bread and wheat bread during storage. Sourdough bacteria were not seen to influence staling. Starch retrogradation during storage was studied by NMR, X-ray diffractometry, and microscopically. ^{13}C NMR results show that the chemical structure of rye and wheat amylopectin and amylose content are very similar; differences were found in the starch phospholipid fraction. The degree of crystallinity of starch in breads, measured by X-ray diffraction, decreased with

hydration and baking down to 3% and increased during 11 days of storage up to 21% in rye sourdough bread and up to 26% in wheat bread. ^{13}C CP-MAS NMR spectra demonstrate that starch in rye sourdough breads crystallize in different forms than in wheat bread. We propose that re-crystallization of starch amylopectin and amylose in a hydrated form causes a decrease in the availability of water during storage and the development of a dry mouth feel during consumption. This effect is less pronounced in rye sourdough bread than in wheat bread due to matrix effects.

KOKKUVÕTE

Kuigi rukkileiva kvaliteedi ja tootmisprotsessi uuringuid on teostatud juba pikka aega, on mitmed tegurid, mis määravad nii rukkijuuretise kääritamist kui ka leivakvaliteeti, siiani ebaselged. Antud töös teostatud kirjeldava sensoorse analüüsi tulemused näitavad, et rukkileib säilib värskena kauem võrreldes nisuleivaga. Käesolev dissertatsioon käsitleb rukkileiva tootmisega seotud tähtsaid aspekte, mis mõjuvad leiva kvaliteeti, toiteväärtust ja säilivust. Antud töös uurisime individuaalsete protsessiparameetrite mõju rukkileiva kvaliteedile ja säilivusele väljatöötatud rukkileiva valmistamise mudelprotsessis.

Juuretise mikroobne kooslus mõjutab suuresti rukkileiva lõppkvaliteeti. Erinevate juuretisebakterite mõju ja kääritamise vältel toimuvate protsesside uurimiseks kasutasime nii kromatograafilisi meetodeid kui ka isotermilist mikrokalorimeetriat. Viimast rakendati eesmärgiga võrrelda kommertsiaalsete starterkultuuride ning tööstuslikest- ja spontaanselt kääritatud juuretistest eraldatud piimhappebakterite kasvukarakteristikuid rukkitaignas. Soojuse eraldumise profiilide ja vaheühendite (lenduvad ühendid, suhkrud, orgaanilised happed ja vabad aminohapped) tekke alusel kääritamisel järeldame, et kuna süsiniku- ja lämmastikuallikate varud ei ammendu juuretise tsükli lõpuks, on happetaluvus määrav tegur, mis mõjutab mikroobikonsortsiumi väljakujunemist juuretise uuendamise tsüklis. Huvitav on ka märkida, et tööstuslikus juuretisetsüklis prevaleerisid hapnikutundlikud mikroorganismid.

Uurisime ka valmistamisprotsessi mõju veeslahustuvate B-kompleksi vitamiinide ning kiudainete sisaldusele leivas. Tiamiini, nikotiinhappe, püridoksiini ja riboflaviini kaod küpsetamisel olid keskmiselt 40%. Nikotiinamiidi sisaldus suurenes aga mikroobide elutegevuse tõttu taignas kümnekordselt. Lahustuvate ja lahustumatute kiudainete suhe suurenes rukkijuuretise kääritamisel ning suurenes ka üldine kiudainete hulk. Leivatootjad peaksid loetletud muutusi arvestama toote pakendite märgistamisel, eriti juhul kui funktsionaalsete ühendite sisaldust leivas arvutatakse retsepti alusel.

Viimases uurimisetapis uurisime me nii rukki- kui nisuleiva säilivust mõjutavaid tegureid. Erinevad juuretisebakterid ei avaldanud mõju leiva vananemisastmele. Tuuma magnettresonantsi (NMR), röntgendifraktomeetria (XRD) ning polarisatsioon valgusmikroskoopia abil uurisime tärklise retrogradatsiooni rukki-ja nisuleibades. ^{13}C NMR analüüsi tulemused näitavad, et rukki ja nisu amülopektiini keemiline struktuur ja amüloosi sisaldus on sarnased; märkimisväärseid erinevusi leiti tärklise fosfolipiidide fraktsioonis mõõdetuna ^{31}P NMR abil. Tärklise kristallisatsiooni aste, mida mõõdeti röntgendifraktomeetria abil, vähenes taigna tegemisel ja küpsetamisel hüdratatsiooni ja geelistumise tõttu kuni 3%-ni ning suurenes 11 päeva kestval säilitamisel kuni 21%-ni rukkileivas ja kuni 26%-ni nisuleivas.

^{13}C CP-MAS NMR spektrid näitasid, et vananenud rukkileivas sisalduv tärklis kristallub erinevates vormides võrreldes nisuleivas sisalduva nisutärklisega. Me järeldame, et tärklise amülopektiini ja amüloosi rekristallisatsioon hüdreeritud vormides vähendab maat-

riksist vee kättesaadavust ning põhjustab “kuiva suu” tunde vananenud leiva tarbimisel, mis väiksema vee sisalduse tõttu avaldub enam nisuleiva puhul.

LIST OF PUBLICATIONS

The following publications form the basis of this dissertation and are reproduced in the appendices with permission from the publishers.

I Mihhalevski A, Sarand I, Viiard E, Salumets A, Paalme T. **Growth characterization of individual rye sourdough bacteria by isothermal microcalorimetry.** *Journal of Applied Microbiology*, 110(2): 529-540, (2011)

II Kaseleht K, Paalme T, Mihhalevski A, Sarand I. **Analysis of volatile compounds produced by different species of Lactobacilli in rye sourdough using multiple headspace extraction.** *International Journal of Food Science and Technology*, 46(9): 1940-46, (2011)

III Viiard E, Mihhalevski A, Rühka T, Paalme T, Sarand I. **Evaluation of the microbial community in industrial rye sourdough upon continuous backslopping propagation revealed *Lactobacillus helveticus* as the dominant species.** *Journal of Applied Microbiology*, (Accepted September 7th, 2012)

IV Mihhalevski A, Nisamedtinov I, Hälvin K, Ošeka A, Paalme T. **Stability of B-complex vitamins and dietary fiber during rye sourdough bread production.** *Journal of Cereal Science*, (In press, available online 22nd October, 2012)

V Mihhalevski A, Paalme T, **Application of isothermic calorimetry and dynamic viscosimetry in studies of rye bread staling.** *Proceedings of the 4th International Congress on Flour - Bread*, 7: 451-455, (2008)

VI Mihhalevski A, Heinmaa I, Traksmaa R, Pehk T, Mere A, Paalme T. **Structural changes of starch during baking and staling of rye bread.** *Journal of Agricultural and Food Chemistry*, 60(34): 8492-8500 (2012)

VII Tohver M, Kann A, Taht R, Mihhalevski A, Hakman J. **Quality of triticale cultivars suitable for growing and bread-making in northern conditions.** *Food Chemistry*, 89(1): 125-132, (2005)

SUMMARY OF AUTHOR'S CONTRIBUTION

I In Publication I, the author performed the experimental work to characterize of isolated rye sourdough bacteria and interpreted the HPLC and isothermal microcalorimetry data. She wrote the paper and is the first and the corresponding author.

II In Publication II, the author performed the experimental work and interpreted the data concerned isolated rye sourdough bacteria characteristics and is co-author of the publication.

III In Publication III, the author performed experimental work concerned rye sourdough characterization, participated in interpreting the results and is co-author of the publication.

IV In Publication IV, the author performed the experimental work, interpreted the results and is the first and the corresponding author.

V In Publication V, the author performed the experimental work, interpreted the results and is the first and the corresponding author.

VI In Publication VI, the author performed the experimental work, interpreted the data, and is the first and the corresponding author.

VII In Publication VII, the author performed the experimental work, interpreted the results and is co-author of the publication. The author's master thesis entitled "Evaluation of baking properties of triticale lines grown in Estonia" was partially taken as the basis for this publication.

LIST OF PRESENTATIONS

I Mihhalevski A, Paalme T. **Application of isothermic calorymetry and dynamic viscosimetry in studies of rye bread staling.** *4th International Congress on Flour - Bread 2007 / 6th Croatian Congress of Cereal Technologists*, October 24-27, 2007, Opatija, Croatia.

II Mihhalevski A, Paalme T. **Study of rye bread staling.** *ICC International Conference Bosphorus 2008*, Aprill 24-26, 2008, Istanbul, Turkey.

III Mihhalevski A, Paalme T, Heinmaa I, Traksmaa R. **The study of rye bread staling.** *7th European Young Cereal Scientists and Technologists Workshop*, May 19-21, 2008, Kaunas, Lithuania.

IV Mihhalevski A, Paalme T. **Investigation on structural changes during storage of rye bread.** *13th ICC Cereal and Bread Congress Cerworld*, June 15-18, 2008, Madrid, Spain.

V Mihhalevski A, Sarand I, Viiard E, Salumets A, Paalme T. **Characterization of bacterial growth in rye sourdough using isothermal microcalorimetry.** *4th International Symposium on Sourdough*, October 14-17, 2009, Freising, Germany.

VI Mihhalevski A, Hälvin K, Ošeka A, Paalme T. **Evaluation of nutritional composition of raw material and rye baking products.** *5th Baltic Conference of Food Science and Technology* FOODBALT-*2010*, October 29-30, 2010, Tallinn, Estonia.

VII Mihhalevski A. **Stability of nutritional composition of rye bread during baking process.** *International Conference "More Attention to Rye"*, October 6-8, 2011, Tartu, Estonia.

VIII Mihhalevski A. **Study of nutritional composition of raw material and rye baking products.** *Flavoure Conference "New Methods in Assessment of Food/Feed Quality and Safety"*, January 17-18, 2012, Saku, Estonia.

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ACRONYMS

API® 50 CH	carbohydrate fermentation strips (bioMérieux, Inc., France)
CFU	colony forming unit
CP-MAS NMR	cross-polarization MAS NMR
DF	dietary fiber
DGGE	denaturing gradient gel electrophoresis
DM	dry matter
DP	degree of polymerization
16S rDNA	small subunit ribosomal ribonucleic acid gene method
GC	gas chromatography
HMW	high molecular weight
HPLC	high pressure liquid chromatography
IMC	isothermal microcalorimetry
LAB	lactic acid bacteria
LC	liquid chromatography
MAS	magic angle spinning
MRS	Agar growth media that favours the growth of *Lactobacilli*, developed in 1960 by de Man, Rogosa and Sharpe.
MS	mass-spectrometry
MW	molecular weight
NMR	nuclear magnetic resonance
PCA	principal component analysis
PLS-R	partial least squares regression
Rep-PCR	repetitive element palindromic polymerase chain reaction
RNA	ribonucleic acid
RPM	revolutions per minute
SDF	soluble dietary fiber
SPME	solid phase micro-extraction
TTA	total titratable acidity
UV	ultraviolet
XRD	X-ray diffraction

CHEMICALS AND ENZYMES

AccQ·Fluor	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
B1	thiamine + TMP + TDP + TTP (vitamin B1 pool), PubChem: 1130
B2	riboflavin + FMN + FAD (vitamin B2 pool), PubChem: 493570
B3	nicotinic acid + nicotinamide + NAD + NADP (vitamin B3 pool), PubChem: 938
B5	pantothenic acid (vitamin B5), PubChem: 6613
B6	pyridoxal + pyridoxine + pyridoxamine + their respective 5´-phosphate forms (vitamin B6 pool), PubChem: 1054
B7	biotin (vitamin B7 or vitamin H), PubChem: 171548
B9	folic acid + folate (vitamin B9 pool), PubChem: 6037
CoA	coenzyme A, PubChem: 87642
DMSO	dimethyl sulfoxide, PubChem: 679
DNA	deoxyribonucleic acid, PubChem: 44135672
FAD	flavin adenine dinucleotide, PubChem: 643975
FMN	flavin mononucleotide, PubChem: 643976
HPMC	hydroxypropyl-methylcellulose (E464)
NAD	nicotinamide adenine dinucleotide, PubChem: 5893
NADP	nicotinic acid adenine dinucleotide phosphate, PubChem: 25147425
NADPH	reduced dihydronicotinamide-adenine dinucleotide phosphate, PubChem: 5884
NADP+	oxidized dihydronicotinamide-adenine dinucleotide phosphate, PubChem: 5884
NADH	coenzyme I, reduced, PubChem: 439153
NAD+	coenzyme I, oxidized, PubChem: 439153
PLP	pyridoxal phosphate, PubChem: 1051
TDP	thiamine diphosphate, PubChem: 1132
TMP	thiamine monophosphate, PubChem: 1131
TTP	thiamine phosphate, PubChem: 15938964

THESIS

1

INTRODUCTION

Hundreds of different commercial sourdough breads are consumed in Europe. They differ in the type of flour, other ingredients used, and the fermentation process and technology used to make them.

Rye sourdough bread is a traditional food in the Baltic states, Denmark, Germany, Poland, and in the European part of Russia; Its common consumption contributes to a shared cultural identity. The continued popularity of rye bread can partially be attributed to its original interesting flavor and nutritional functionality. Rye breads are made from rye sourdough using various recipes in large factories, artisan bakeries, and at home, thus giving a wide variety of rye breads. Rye bread processing includes rye sourdough fermentation, leavening, and baking. Frequently, scalding is also applied.

The structure of rye sourdough bread differs from wheat bread due to a lack of gluten and the importance of pentosans in dough structure formation. To achieve the distinctive structure of rye bread, most production schemes include sourdough fermentation which is not as important in determining the structure of wheat bread. Rye bread maintains its quality longer than wheat bread which explains why rye bread was prepared in farmhouses only once every week or two. The stability of rye bread during storage was a historically important factor compensating the more complex technology of rye bread baking compared to that of wheat bread. This practical aspect helped to maintain the tradition of baking rye bread.

Compared to the worldwide production of wheat (695,000 million tons in 2011/2012), rye production is quite moderate (12,800 million tons) according to the FAOStat database [1]. The amount of rye used for food and bread making compared to wheat follows the same ratio. The production of rye reduced by about 2 times during the last 20 years while rye supplied as a food has remained constant during the same period [1]. This suggests that rye products, and particularly rye sourdough bread, continue to be consumed as a traditional food.

Modern consumers are aware of the nutritional composition and health benefits of rye bread which include it being a good source of dietary fiber and water soluble vitamins. This knowledge, together with cultural inheritance, supports the consumption of rye sourdough bread and the expansion of rye sourdough consumption outside of traditional areas.

In contrast to wheat bread, rye bread processing almost always involves sourdough fermentation, and in several countries such as Estonia, also scalding. The sourdough fermentation process acidifies the dough which supports rye bread structure formation by increasing both swelling and the solubility of pentosans [2, 3]. The lower pH also

inhibits arabino-xylanase activity [4]. Although frequently used in the production of wheat bread, sourdough fermentation is not necessary for structure formation due to high gluten content in wheat flour. In addition to lowering the acidity level, lactic acid fermentation of flour contributes to the flavor of bread through the formation of volatile aromatic compounds. Adding scalded flour and yeast to sourdough before leavening supports enhanced flavor development within rye bread. The saccharides (maltose and maltodextrins) formed from starch during scalding provide additional substrates for the yeast during fermentation.

Usually, the amount of nutritional components in rye bread is reported based on the composition of the ingredients used to produce the bread. However, rye bread making is a complex process with its use of sourdough fermentation, scalding of the flour, mixing with other flour (rye and wheat), adding yeast and other components, leavening, and baking. These processes, together with natural variations of nutrients in biomass, affect both the precision and accuracy of estimating the nutritional composition of the finished product based on the composition of the ingredients. The biochemical changes that occur during bread processing affect the content of vitamers and fibers.

There have been attempts to combine the good baking properties of wheat with the nutritional properties of rye. Triticale, the intergeneric hybrid between wheat and rye, displays good growth yields, however, it has moderate baking properties [5], (Publication VII) due to a lower quantity of pentosans than that in rye [6] and weaker gluten than that in wheat [5].

Despite the long history of rye sourdough bread production the biomolecular and physical processes that occur during processing and storage are poorly understood.

One major problem faced by the rye bread processing industry is the stability of the rye sourdough fermentation cycle; Variations in the microbial consortia of sourdough affect the sensory properties of bread. However, the most critical commercial production objective is staling. Although rye bread maintains its sensory properties better than wheat bread, staling remains a big issue.

A consistent quality of sourdough used for bread making has been achieved by carrying out the sourdough fermentation using regular backslopping over the years. Scalding has also been applied to improve the flavor, structure, and shelf life of rye bread.

This study provides insight into several crucial steps of this technologically complex process that affect the quality of the bread produced. The stability of the sourdough composition, both microbial and chemical, is the most important factor affecting the bread quality. Preservation of sapor and freshness are important factors in bread quality during bread storage. The high acidity of sourdough breads has been suggested to inhibit both bread staling and mold growth during storage. Bread staling has been mainly related to starch retrogradation [7, 8], however, prior to this research there was a gap in the literature relating starch retrogradation mechanisms to differences in staling rates and quality degradation between rye sourdough and wheat breads. Using modern instrumental methods such as XRD and NMR, together with other methods, we studied starch structural changes and their role in rye bread staling.

The experimental part of this doctoral work was performed at (I) the Competence Center of Food and Fermentation Technologies (microcalorimetry, rheology, sensory analysis, HPLC, GC-MS, LC-MS, microbiological studies), (II) the Department of Food Pro-

cessing of Tallinn University of Technology (microscopy, texture), (III) the Department of Materials Science and Centre for Materials Research at Tallinn University of Technology (crystallinity studies, XRD analyses), and (IV) the National Institute of Chemical Physics and Biophysics (NMR analyses).

2

LITERATURE REVIEW

The overview presented below focuses on the elucidation of the biological, chemical and physical processes that occur during bread making that affect the rate of quality degradation during storage.

2.1 SOURDOUGH FERMENTATION

Dough fermentation and the production of sourdough breads can be traced back to ancient times. Most probably the first leavened breads were sourdough breads. Sourdough can be defined as a mixture of flour and water that is fermented with lactic acid bacteria and yeast. Consecutive microbial re-inoculation of the microorganisms from a previous batch of the sourdough cycle, termed "backslopping", is used to maintain the microbial flora, which adapts and select to the applied process conditions [9]. Individual organisms that form the sourdough consortia originate from the starter culture, flour, fermentation vessel, and the natural environment, including humans. A reproducible and controlled composition of the sourdough, with stable activity is required to achieve consistent bread quality [10].

Sourdough fermentation enhances dough properties by increasing the volume, improving the texture, flavour, and the nutritional value of the bread. Fermentation retards the staling process of bread and protects bread from mould and bacterial spoilage [11–13]. The low pH of rye sourdough increases the solubility and swelling properties of rye pentosans and inactivates amylolytic and xylanolytic activity. These processes contribute to an increase in the capacity of bread to bind water [3] and influence rye bread structure during leavening and baking. Those peculiarities partially explain why rye bread prepared with sourdough stales slower than wheat breads [2].

Sourdough is classified into 3 types [10, 14]: Type I is mostly used in small-scale bakeries. It has a pH of about 4.0 and is fermented between 20°C - 30°C. Type II is used in large-scale industrial bread processes which employ temperature above 30°C and typically achieve a pH of 3.5. Type III is initiated with a drying tolerant LAB strains belonging to the species *Lactobacillus brevis* and *Lactobacillus plantarum*. It is difficult to clearly classify between type I and type II sourdough mixes in Estonian bakeries, while type III are practically not used in Estonia.

Adaptations of bacteria to a specific sourdough environment might include (I) a unique central metabolism and/or transport of sourdough-specific carbohydrates such as maltose and fructose, (II) an activated proteolytic activity and/or arginine deiminase pathway;

(III) particular stress responses; and (IV) production of antimicrobial compounds [13]. As a result of the selective pressures exerted by the environmental conditions, cereal fermentations are characterized by a comparatively stable association of Lactobacilli even in aseptic conditions [15].

Rye domestic microflora may play a significant role in the formation of microbial consortia within the rye sourdough cycle. Rye flour may contain bacteria in quantities from 10 to 10^7 CFU·g^{-1}, with LAB constituting only between 10^2 to 10^3 CFU·g^{-1} [16, 17]. LAB that become dominant in the sourdough cycle are strictly fermentative, acidophilic, salt-tolerant, and have complex nutritional requirements for carbohydrates, amino acids, peptides, fatty acids, salts, nucleic acids derivatives, and vitamins [18–20].

The technological parameters of the rye sourdough cycle differ significantly between bakeries and thus cause variations in the microbial consortia of sourdoughs [21, 22]. The most reliable and simpler method of initiation of dough fermentation is used in commercial starter cultures to control specific properties [10]. For example, starters are used to control gas formation ability, aroma, taste, rheological properties (IsernHäger and Co. KG), or for specific bread recipes (rye-wheat mixed bread, mixed wheat bread, pastries etc. (Ernst Böcker GmbH and Co. KG; Lallemand Inc.). However, commercially available strains are not stable over a long term sourdough cycle and often cannot compete in the long run with endogenous microflora in the flour introduced during renewal cycles [23, 24].

To understand the mechanisms that determine dominance of particular bacterial species in repeatedly backslopped sourdoughs, the bacteria should be studied in their natural growth environment as the growth characteristics determined in artificial liquid media and even in flour extracts might be misleading.

Evaluation of the growth characteristics of individual bacterial strains in sourdough is a complicated task because no precise method exists for microbial biomass quantification in both opaque media and in solid matrixes. The method of plating out might not be quantitative or suitable for several species present in industrial sourdough [13]. In complex food matrices, applying indirect methods such as measuring the total bacterial DNA, RNAs, proteins [25] or the concentration of cell wall components, is often prohibitively complicated. Alternatively, measuring metabolite concentrations and CO_2 formation are often used to estimate microbial growth during solid-state fermentation [26]. Although the growth yields may depend significantly on the growth conditions, parameters such as oxygen consumption, CO_2 formation, and the production of acid and heat provide valuable indicators for continuous on-line measurement of the microbial growth in the solid state.

Metabolic processes of bacterial cells are accompanied by heat production which can be monitored continuously using isothermal microcalorimetry (IMC) [27–29]. In IMC the net heat flow generated by any biological or non-biological chemical or physical processes taking place within sourdough can be continuously measured at a constant temperature in 4 - 96 parallel experiments, depending on the instrument. The measurement of different growth parameters in parallel with the heat production rate has made it clear that the shape of the power–time curve is influenced by the type of metabolic activity and can be related to different physiological states of bacteria [27, 28, 30].

To measure the growth of individual species in rye sourdough, culture independent DNA based methods such as DGGE [23] and pyrosequencing [31] can be used. However, the precision of DNA methods is insufficient for dynamic studies of microbial fermentation. To study the stability of sourdough during a long sourdough cycle, a combination of DNA based culture dependent and culture independent methods can be successfully applied (Publication III).

The dominating species of rye sourdough microbial consortia are commonly presented as both homofermentative and heterofermentative LAB [15]. The heterofermentative species produce lactate, ethanol, and acetate which are considered important for the development of important sensory properties of rye breads. For the growth of LAB, sugars (glucose, maltose, fructose etc.) act as sources of carbon, and amino acids or short peptides and several vitamins are essential. Rye flour is a good source of these components. It contains 0.2-0.8 $mg{\cdot}100g^{-1}$ glucose, 0.3-0.6 $mg{\cdot}100g^{-1}$ fructose, 1.0-3.4 $mg{\cdot}100g^{-1}$ maltose, about 9 $mg{\cdot}100g^{-1}$ amino acids, and for vitamins between 280-330 $\mu g{\cdot}100g^{-1}$ B1, 130-200 $\mu g{\cdot}100g^{-1}$ B2, 400-1300 $\mu g{\cdot}100g^{-1}$ B5, and 160-400 $\mu g{\cdot}100g^{-1}$ B6 [32–34]. In addition, flour enzymes are able to deliver additional sugars and amino acids/peptides necessary for fermentation. The question arises if proteolytic and amylolytic activity provides a growth advantage for LAB in the sourdough cycle.

2.2 RYE SOURDOUGH BREAD AS SOURCE OF VITAMINS AND FIBER

Rye bread is a recommended part of our daily diet because it is a good source of various biologically active substances, including dietary fiber and B-group vitamers [35–37] due to the high extraction rate of rye flour [35, 38] and the products of lactic acid fermentation [17, 36]. Typically, commercially produced rye breads report the content and composition of vitamins and fiber based on the composition of the ingredients used to make the bread. However, this may result in incorrect estimations, mostly due to variations in the vitamins and fiber content, but also due to biochemical changes that occur during processing. The enzymatic activities of flour and lactic acid bacteria, as well as thermal treatment applied during rye bread baking, may also affect the concentration of dietary fiber and B-group vitamers in rye bread.

2.2.1 *Water soluble B-complex vitamins in bread*

The vitamins in bread originate mainly from the raw materials. The extent of *de novo* vitamin synthesis by LAB and yeast during sourdough fermentation is not known, however, the ability of LAB to synthesize the *de novo* vitamins is limited. In contrast, baker's yeast is auxotrophic only for biotin. The term vitamin refers to a number of vitamer compounds with the same or similar biological activity as the respective vitamin. Unfortunately, the list of vitamers in food is not complete. Several vitamers are derived from cofactors as the result of enzymatic activities during food processing, storage, and digestion in both the food matrix and digestive tract [39, 40]. The efficiency of this conversion varies significantly. In addition, some vitamers such as thiamine, riboflavin, nicotinic acid, pantetheine, and pyridoxine which are very stable to degradation under physiological

conditions can be inactivated during rye bread baking. On the other hand, the concentration of some vitamers can increase at the expense of co-factors present in the raw material due to enzymatic and microbial activities of flour and sourdough bacteria

Vitamin B1 in cereals is present naturally as thiamin (90%) and its phosphorylated forms (Figure 1) thiamine monophosphate (TMP), thiamine diphosphate (TDP) (2 - 10% of the vitamin B1 pool) and thiamine triphosphate (TTP) [41]. Thiamine is relatively stable at pH 2.0 – 4.0 up to the boiling point [38, 42]. Phosphorylated forms of thiamine in flour are more thermolabile [43]. The loss of B1 due to thermal breakdown during cooking and baking can reach 20 - 50% [38, 44, 45]. Also, lactic acid fermentation can decrease the thiamine content [43].

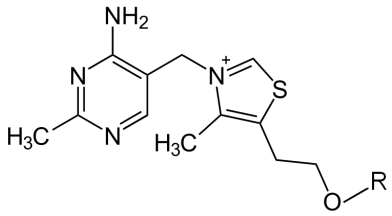
The average rye kernel contains between 280 – 400 $\mu g \cdot 100 g^{-1}$ of thiamine hydrochloride equivalents [32, 33] depending on the rye variety and growth conditions, especially soil type [44].

Vitamiin B2 is mostly present as riboflavin, flavin mononucleotide (FMN), and flavin adenin dinucleotide (FAD) (Figure 1). Riboflavin can also be found in glycosylated forms in some plants [46]. FMN and FAD can exist in free or protein bound states [42]. Riboflavin is relatively heat-stable [43]. It has been reported that whole wheat dough fermentation in the presence of yeast can enhance the content of riboflavin by up to 30%, while sourdough lactic acid fermentation without yeast did not result in any increase in the bread´s riboflavin content [43].

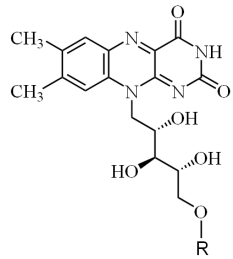
Vitamin B3 is mostly present in food as nicotinic acid, nicotinamide, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP) (Figure 1) and can be also synthesized *in vivo* from L-tryptophan. In mature cereal grains 85–90% of the total B3 content exists in cofactor and tryptophan form, with the remainder being present as free nicotinic acid and nicotinamide [47, 48]. The data on vitamin B3 activity of "bound" forms are contradictory. According to Wall and Carpenter [49], bound forms of nicotinic acid are not biologically available after cooking and only a small part of NAD can be hydrolyzed by gastric juice into bioavailable forms. Sourdough fermentation can increase the availability of bounded niacin subunits via acidification and enzymatic hydrolysis. Nicotinic acid is normally stable during baking [45].

Vitamin B5. The simplest vitamer in the vitamin B5 pool is pantothenic acid (Figure 1), which is a component of coenzyme A. Pantothenic acid has good heat stability at pH 4.0-5.0 [42, 45, 47]. On the other hand, pantothenic acid contains a linkage which is unstable under acid conditions and thus the stability during rye sourdough baking might be lowered from that of wheat bread.

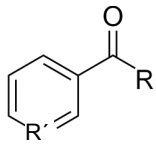
Vitamin B6 consists of six closely related derivatives of 2-methyl-hydroxypyridine including pyridoxal, pyridoxine, pyridoxamine, and the respective 5´-phosphate forms. In addition, some foods of plant origin may contain β-glycosylated forms of pyridoxine [50]. In cereal grains, over 90% of the vitamin B6 is found in the bran and germ, and 75–90% of the B6 content of the whole grain is lost in the milling of wheat to low extraction flour. Pyridoxine is more oxygen and heat tolerant than pyridoxal and pyridoxamine [51]. During bread processing about two thirds of the pyridoxine pool is lost [43].



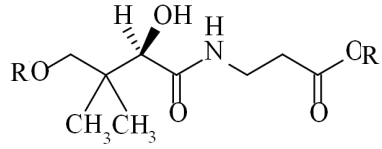
Thiamine, R = H
TMP, R = PO_3H_2
TDP, R = $P_2O_6H_3$
TTP, R = $P_3O_9H_4$



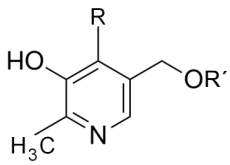
Riboflavin, R = H
FMN, R = PO_3H_2
FAD, R = adenosin diphosphate
Riboflavin glucoside, R = $C_6H_{11}O_5$



Nicotinic acid, R = OH, R′= N
Nicotinamide, R = NH_2, R′= N
NAD, R = NH_2, R′= N^+ adenine dinucleotide



Pantothenic acid, R = H, R′= COOH
Acetyl-CoA, R = adenosine triphosphate, R′= HS
Pantetheine, R = COOH, R′= HS



Pyridoxine, R = CH_2OH, R′= H
Pyridoxal, R = CHO, R′= H
Pyridoxamine, R = CH_2NH_2, R′= H
Pyridoxal- 5´- phosphate, R = O, R′= H_2PO_3
Pyridoxine glucoside, R = CH_2OH, R′= $C_6H_{11}O_5$

Figure 1 – Structures of water-soluble B-complex vitamins. The vitamers with restricted vitamin activity in humans are given in bold gray.

Vitamin B7. Biotin content in rye and wheat whole meal flours is 6-7 $\mu g \cdot 100g^{-1}$ and 1.9 $\mu g \cdot 100g^{-1}$, respectively. Rye whole meal bread contains 3.40 – 5.90 $\mu g \cdot 100g^{-1}$ [32, 33]. The recommended daily intake is 20-35 $\mu g \cdot day^{-1}$ [52]. It is stable in solution at pH 4.9 [42].

Vitamin B9. Rye is also known as a source of folic acid and folate (B9). Folates (reduced metabolites of pteroylmonoglutamic acid) are a group of B9 vitamins responsible for many human metabolic functions and cannot be sunthesized by humans [53]. The recommended daily intake of folate is 200- 500 $\mu g \cdot day^{-1}$ and depends on age, the stage of pregnancy, and lactation [52]. Rye whole meal flour contains 60 – 85 $\mu g \cdot 100g^{-1}$ folate [32, 33, 53]. Kariluoto and coauthors [54] reported the contribution of yeast in folate synthesis during rye sourdough fermentation. At the same time some folate can be utilized by lactic acid bacteria. Baking losses of ~ 25% have been observed [54]. Frozen storage of rye bread can also reduce the folate concentration [53].

2.2.2 *Dietary fiber*

Dietary fiber (DF) is defined as an indigestible complex of carbohydrates and lignin [55]. The health effects of DF consist in reducing of risk of coronary heart disease, diabetes, obesity and some forms of cancer [55–57]. The recommended daily intake of dietary fiber is 25 $g \cdot day^{-1}$ for adult females and 38 $g \cdot day^{-1}$ for adult males [58].

The most commonly used methods for the determination of dietary fiber in cereals are the enzymatic-gravimetric methods [59, 60] and the neutral sugar residue, uronic acid residue, and Klason lignin method, also known as the Uppsala method [61]. The dietary fiber content of typical rye bread (16 – 18 $g \cdot 100g_{DM}^{-1}$) is about three times higher than that of white wheat bread (3.5 – 4.2 $g \cdot 100g^{-1}$) [32, 33, 62–64]. The major reason for this is that rye bread is usually made from whole grain rye flour, while white wheat bread is typically made from refined wheat flour.

Rye grain contains various fiber compounds including arabinoxylans (8 – 12%), fructans (4.5 – 6.4%), β-glucans (1 – 3%), and lignin (1 – 2%) [65–67]. The soluble (water extractable) fibers (SDF) present in rye grain are fructo-oligosaccharides 1-kestose (0.6%), 1,1-kestotetraose (0.3%), and 1,1,1-kestopentaose (0.3%) [67], water-extratable β-glucans (1.2%) [68] and arabinoxylans (3.95 – 5.4%) [69]. Soluble dietary fibers can act as prebiotics, providing food for probiotic bacteria in the large intestine and colon [70, 71].

Enzymatic as well as thermal treatment can modify the ratio between soluble and insoluble fibers during bread making [72, 73]. Already during flour hydration several intrinsic hydrolytic enzymes of cereals are activated [62, 74]. Also, enzymes with β-glycolytic activity from lactic acid bacteria [75] and yeast [76] can affect the fiber composition during rye bread processing [63]. The degree of hydrolysis of fibers affects the water binding and gas holding capacity of bread dough as well as bread structure and behavior during storage [2, 77].

2.3 PHYSICAL PROCESSES IN BREAD DURING BAKING AND STORAGE

2.3.1 *Role of starch in bread staling*

Bread after baking is a biochemically inactive multi-component food system in which the sensory parameters reach an optimum shortly after cooling. Bread quality decreases after this point with crumb firmness increasing and fresh flavours are replaced by ones associated with stale bread. The rate of bread staling depends on the recipe, technological process employed, and the storage conditions.

Although the nutritional composition of wheat and rye is quite similar, the bread making properties of those flours differ significantly. In contrast to wheat gluten, rye proteins (secalins, prolamins) as well as triticale secalins and high molecular weight (HMW) glutenin subunits [78] cannot take part in the formation of starch-protein complexes that have high gas retention. The function of gluten in rye sourdough bread is partly taken over by water extractable pentosans (arabinoxylans). The optimal ratio of water extractable pentosans (arabinoxylans) to starch in rye flour is 1:20 to form an acceptable rye dough [79]. Viscosity-enhancing, water-holding ability and the gelatinization potential of arabinoxylans and starch are important in both the baking and staling processes [77, 79, 80]. The interaction of pentosans with starch has been suggested to play an important role in retarding the starch retrogradation process [81]. Under low pH the ability of pentosans to absorb water is enhanced. Enzyme activities are inhibited and the water-binding capacity of starch is also enhanced [2]. A higher amount of dietary fiber in rye sourdough bread than in wheat bread can explain the initial hardness of rye bread [79]. At the same time, pentosans are suggested to act as plasticizers retarding starch retrogradation and reducing staling [82]. The degree of hydrolysis of fibers affects the water binding and gas holding capacity of bread dough as well as bread structure and behavior during storage [2, 77].

The main factor which limits the shelf life of breads is staling, which is generically a physical process in bread and other starch containing foods that reduces their palatability. The precise mechanisms of staling are incompletely understood, and most theories are related to changes in starch structure during baking and storage with the migration of water within the macro- and microstructures of bread playing an important role. The rate of staling depends on the bread recipe and storage conditions. Rye sourdough breads contain about 42 – 44% water which is commonly higher than that found in wheat breads (38 – 41%) with a water activity of around 0.95. During storage, water migrates from the crumb to crust, which leads to a decrease in both the water concentration and activity in the crumb [83] and softening of crust [84]. This, however, is not the main reason for staling, which is generally related to starch retrogradation [7, 8]. Other bread components such as gluten in the case of wheat bread, water-soluble proteins, oligosaccharides, and lipids are involved in the staling process [85].

Native starch is presented in cereals and flour as semi-crystalline granules consisting of two types of α-(1→4)-glucan (Figure 2): branched (5-6% of α-(1→6)-linkages) amylopectin and linear amylose with less than 1% α-(1→6) branched linkages [86]. The molecular weight (MW) of amylopectin is 2.6 – 7.0 x 10^5 kDa and the degree of polymer-

ization (DP) more than 10 000 [87, 88]. Amylose MW is 40 – 350 kDa and DP 500 – 5000 [89–91].

The properties of starch, including the ratio of amylose to amylopectin and water content, depend on its botanical origin. The average content of amylose is 22 - 24% in rye starch [90], which is a lower range than that reported in wheat starch (23 - 28%) [87, 92]. The molecular weight of rye amylose is lower than that of wheat amylose (220 kDa versus 260 kDa, respectively) [93]. The structure of the rye amylopectin is reported to differ from that of wheat; Wheat amylopectin displays a higher β- amylolysis limit, longer average chain length, longer external chain length, and shorter internal chain length than rye amylopectin [94]. Those structural peculiarities were related to differences in the gelatinization and retrogradation parameters of wheat and rye starch.

Starch granules contain radial growth rings comprised of semi-crystalline regions of ~140 nm thick shells separated by an amorphous zone of roughly the same thickness (Figure 2). The semi-crystalline rings contain 16 radiating clusters of amylopectin exterior chains with the actual length of the double helices ~6.65 nm interspersed within amorphous lamellae of about 2.2 nm (amylopectin α-(1→6) branch points) [87, 92, 95–97]. The amylose could be present in the granule amorphous zone either as lipid-complexed amylose or lipid-free amylose [98].

Typical cereal starches such as wheat and rye starches are classified according to XRD pattern as an A-type crystalline structure (Figure 2) where double helices are densely packed in a monoclinic lattice of 12 glucopyranosyl units with 4 water molecules [99]. B-type starch crystals, typically made by tuberous plants, are crystallized in a hexagonal lattice with 27% hydration [87, 99–102] with water molecules being incorporated instead of another double helix structure [102].

After swelling of the starch granules during dough making and gelatinization during baking, the ordered semi-crystalline structure of the starch is disturbed and changed into amorphous starch [103]. During bread cooling and storage the amylose and amylopectin chains realign themselves and the crystalline structure slowly reforms [8, 88, 104, 105]. This process is termed starch retrogradation and is technologically important because of the significant changes in the properties of the crumb which in turn affect its sensory properties [106].

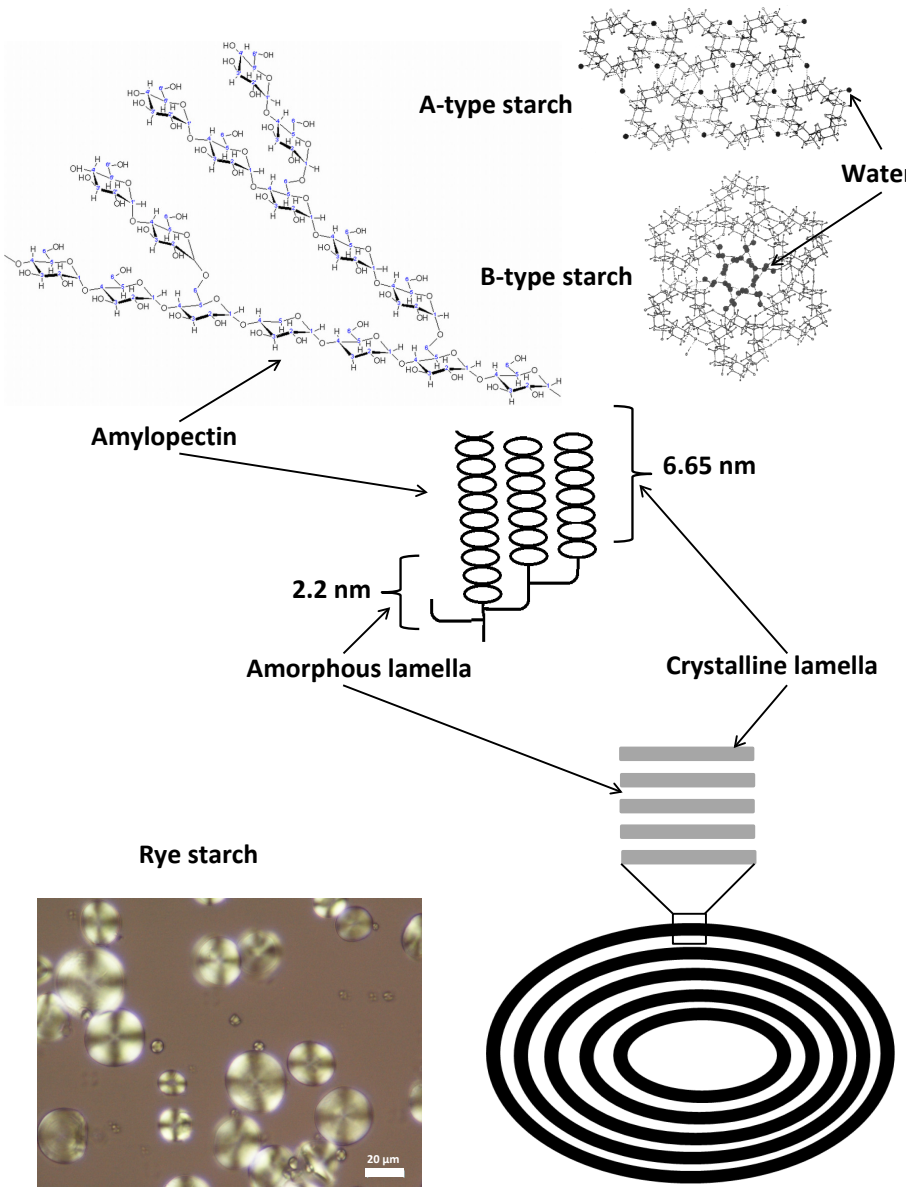


Figure 2 – Structure and types of starch granule . Example of native rye starch granules obtained by polarized light microscope (at the lower left corner) with typical Maltese cross pattern; schematic representation of the starch granules (on the right). Semicrystalline and amorphous growth rings decomposed into amorphous and crystalline lammellae consisted of amylopectin chains. (According to: [92, 95, 100, 101, 105], and Publication VI).

Much effort has been spent attempting to decrease the rate of starch retrogradation effect during bread staling. Guarda and coauthors [107] studied the anti-staling effects of hydrocolloids (0.1 - 0.5% w/w flour basis) sodium alginate and hydroxypropylmethycellulose (HPMC) on wheat bread. Hydrocolloids are able to increase water absorption, holding capacity and decrease the relative dehydration rate of the crumb during bread storage. Although the use of HPMC (E464 according to EU labeling of food additives) and sodium alginate (E401) is permitted in Europe, the maximum level of these additives is limited by EC Regulation (EU No. 1130/2011) as *quantum satis*. It should be noted that the above mentioned compounds or their derivatives are not typically used in Estonian rye bread.

Hug-Iten and coauthors [108] observed that adding α-amylase enhanced the initial firmness of starch gels and reduced the rate of firming of bread during aging. The anti-staling effect of this enzyme is based on the partial degradation of amylopectin, thus hindering its crystallization. They also observed rapid formation of a partly crystalline amylose network and the prevention of amylose rearrangements during aging. Improvement of the shelf-life of wheat bran bread by addition of the enzyme mixture (α-amylase, xylanase, lipase) by altering water migration between starch, protein and bran particles during storage was observed by Katina *et al.* [109].

Addition of glycerol retarded starch retrogradation by affecting the dynamics of the molecular groups around the C6 region and acting as plasticizer [110]. At the same time a rapid increase in bread firmness was observed, what suggests that the firming of bread can be controlled by other events and not solely by starch retrogradation (*i. e.*, local dehydration of the matrix, gluten network stiffening, water distribution and migration).

Scalding - the mixture of flour and hot water that is allowed to cool - is reported to affect the rate of staling of rye bread [7, 111]. Estonian bakeries usually scald 10-20% of the rye flour used for rye bread making and rye or barley malt is added before scalding the flour. This process increases the extent of starch and protein hydrolysis and enhances the formation of aroma and flavor during leavening and baking.

3

AIMS OF THIS DISSERTATION

The main objective of this dissertation is to evaluate the factors that determine the changes in the nutritional and sensory qualities of rye sourdough bread during processing and storage. Specific topics addressed are:

I Development of a laboratory rye sourdough model process.

II Characterization of the technological properties and aroma profiles of sourdough bacteria isolated from industrial, spontaneously fermented laboratory sourdoughs, as well as commercial strains.

III Analysis of the content of B-complex vitamins and dietary fiber in raw materials and breads to evaluate the stability of these compounds during rye sourdough bread processing.

IV Characterization of the structural changes of starch in rye bread during staling in comparison with wheat bread.

4

MATERIALS AND METHODS

More detailed descriptions of the materials and methods applied are available in the publications. The following sections are provided to make this material more accessible.

4.1 MATERIALS

4.1.1 *Microorganisms*

The bacterial strains used in this study were (I) *Lactobacillus plantarum* L73 and *Lb. brevis* L62 obtained as commercial freeze dried preparation from Lallemand Inc. (Montreal, Canada); (II) *Lb. helveticus* N92, *Lb. helveticus* E96, *Lb. casei* N726, *Lb. panis* N915, *Lb. vaginalis* N1113, *Lb. vaginalis* E1113 and *Lb. pontis* N131 isolated from an industrial sourdough cycle (Publication III) and (III) *Lb. sakei* 0E12-10, *Lb. curvatus* 0E12-11, *Leuconostoc citreum* 3N18-10 and *Lb. brevis* 0E12-37 isolated from spontaneously started laboratory rye sourdough (Publication III). Baker's yeast suspension (20% DM) was provided by AS Salutaguse Pärmitehas (Kohila vald, Estonia).

4.1.2 *Ingredients used for bread making*

Whole grain rye flour R1800 (protein 8.6 $g \cdot 100g^{-1}$; FN 140-180 s; fat 1.6 $g \cdot 100g^{-1}$), dark rye flour R1370 (protein 8.2 $g \cdot 100g^{-1}$; FN 170-210 s; fat 1.6 $g \cdot 100g^{-1}$), fine rye flour R705 (protein 6.1 $g \cdot 100g^{-1}$; FN 170-210 s; fat 1.1 $g \cdot 100g^{-1}$), wheat flour W700 (protein 11.8 $g \cdot 100g^{-1}$; FN 250-320 s; fat 1.5 $g \cdot 100g^{-1}$), steel cut rye grains (protein 9.5 $g \cdot 100g^{-1}$; FN 120-150 s; fat 1.7 $g \cdot 100g^{-1}$), were obtained from Tartu Grain Mill Ltd. (Tartu, Estonia). Red malt (protein 7.65 $g \cdot 100g^{-1}$; fat 1.65 $g \cdot 100g^{-1}$; pH 4.0-4.3; Colour EBC 150-220) and white rye malt (protein 11.2 $g \cdot 100g^{-1}$; fat 2.91 $g \cdot 100g^{-1}$, pH 6.0-6.3) were obtained from Eesti Leivalinnase Ltd. (Võru, Estonia).

Sucrose, margarine (fat 80%), milk powder (fat 26%, moisture 4%) and salt for laboratory bread baking (Publication V, Publication VI, and Publication VII) were obtained from a local market. Dark rye flour (R1370) used for incubation and fermentation experiments was irradiated in sealed 400 g plastic Stomacher bags at 10 kGy using a dosimetric system GEX WinDose (Centennial, CO, USA).

4.1.3 *Commercial rye breads*

A commercial rye bread and the raw materials used for its preparation were obtained from a local bakery (Publication IV). Breads for comparative sensory analysis of wheat and rye breads were obtained from a local market.

4.1.4 *Starches*

Wheat, corn, and potato starches, amylose from potatoes, and amylopectin from maize were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Rye starch used to study the structure and its role in rye bread staling was isolated according to Verwimp and coauthors [112] (Publication VI, Publication VII). Rye flour was suspended in tap water and 0.5% NaOH in a 2:7:7 ratio, stirred for 60 minutes, and centrifuged at 3000 RPM for 10 minutes. The sediment was washed with water and centrifuged again. The procedure was repeated 2 times, neutralized with 0.1 N HCl, and centrifuged. The upper grayish layer was removed and the white starch was washed with water and centrifuged again. The isolated starch was air-dried overnight and sieved trough a 250 μm sieve.

4.1.5 *Enzymes*

α-amylase from *Aspergillus oryzae*, β-glucosidase from almonds, and potato acid phosphatase were obtained from Sigma-Aldrich and used for vitamin extraction.

4.1.6 *Reagents*

All reagents and standards used in this study were of analytical grade. More detailed information of the content and suppliers of the reagents used can be found in the corresponding publications.

4.2 METHODS

4.2.1 *Isolation, identification, and characterization of dominant rye sourdough bacteria*

I Isolation and determination of lactic acid bacteria strains from industrial and spontaneously fermented laboratory rye sourdough was made using repetitive element palindromic polymerase chain reaction (Rep-PCR), denaturing gradient gel electrophoresis (DGGE), and small subunit ribosomal ribonucleic acid gene method (16S rDNA) methods.

II The substrate consumption profiles of individual LAB were determined with the aid of API® 50 CH carbohydrate fermentation strips.

III The growth of bacteria in both dough and MRS media was characterized by determining the heat flow using an isothermal microcalorimeter TAM III or optical density in Bioscreen C plates. The dough was inoculated by mixing rye flour with the starter culture suspended in 0.5% NaCl at a 1:1 ratio (10^6-10^7 CFU·ml^{-1}) or with fermented sourdough (backslopping 1:10) in 400 ml plastic bags, mixed for 15 minutes at 100 RPM in a Stomacher 400 circulator.

4.2.2 *Bread making*

A laboratory process of sourdough bread making was developed to simulate industrial conditions. This process was used to study the effect of individual process parameters on bread quality and is illustrated in Figure 3.

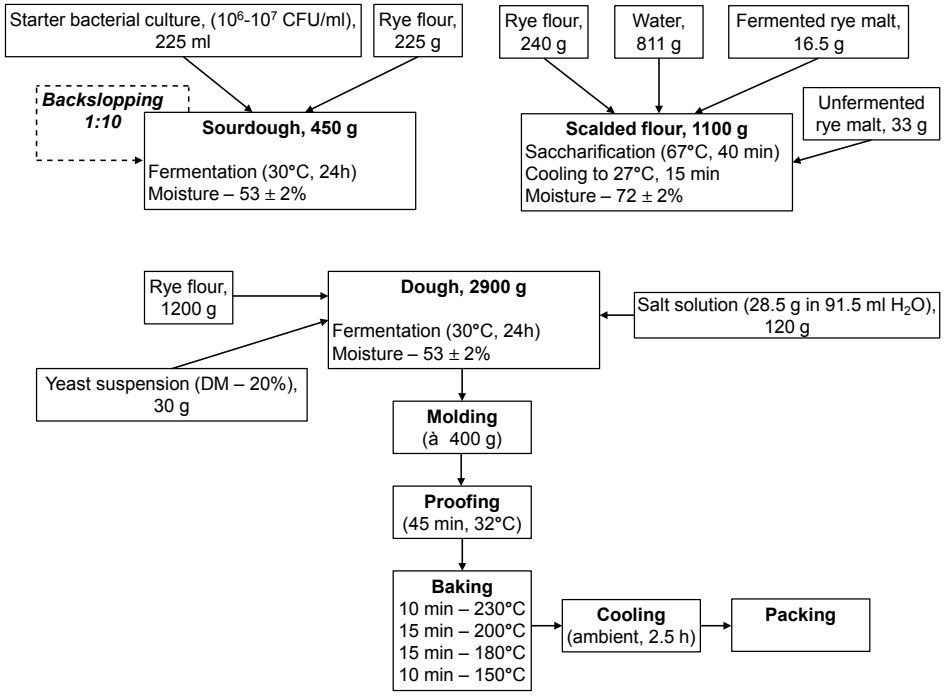


Figure 3 – Laboratory rye sourdough bread process flow diagram.

To prepare the sourdough, 225 g of dark rye flour and a bacterial suspension of the chosen LAB strains (5×10^6 – 5×10^7 CFU·ml^{-1}) in 0.5% of NaCl was mixed in a 1:1 ratio in plastic bags in Stomacher 400 Circulator (Seward Ltd., Worthing, UK) for 15 minutes at 100 RPM, closed hermetically, and then incubated in an Environmental Test Chamber (Sanyo, Japan) at 32°C for 24 hours.

In the scalding process, 239.5 g of rye dark flour was added into 810 ml of warm tap water (55°C) in a 2 L temperature controlled kettle equipped with a refrigerating/heating circulator (Julabo F25, Seelbach, Germany). After 15 minutes of mixing, rye white malt

was added. This mixture was heated to 67°C for 40 minutes to allow for saccharification after which it was cooled down to 27°C.

Leavening was carried out in an Environmental Test Chamber (Sanyo, Japan) at 32°C for 110 minutes. Afterwards the sourdough (450 g) was mixed with scalded flour (1100 g), rye dark flour (1200 g), yeast suspension (30 g, dry weight 20%), salt solution (24%) and mixed for 20 minutes at 100 RPM in a 5 L Bear Teddy dough mixer (Varimixer, Shreveport, LA, USA). The dough was then molded into 400 g portions, put into baking molds (8.5 × 15 × 9.5 cm), and proofed in the Environmental Test Chamber at 32°C for 45 minutes.

Baking of proofed dough pieces was carried out in a Self Cooking Center (Metos System Rational, Weikersheim, Germany) as follows: 10 minutes – 230°C, 15 minutes – 200°C, 15 minutes – 180°C, 10 minutes – 150°C.

Cooling and packaging. The bread was taken out from the oven and cooled down to room temperature in a laminar flow cabinet under UV light (Telstar, Terrassa, Spain), sealed aseptically into plastic bags and stored at room temperature.

Unlike laboratory rye bread process the industrial process includes large scale preparation of liquid rye sourdough (about 35% DM) with constant mixing. The renewing of the industrial sourdough cycle (backslopping) was carried out by mixing a mother sponge, rye flour and water in a 10:36:54 ratio followed by fermentation at 33°C for 10 hours.

4.2.3 *Analytical methods*

I The dry weight of samples was measured by a Halogen Moisture Analyzer HR83.

II The water activity of bread was measured using a Water Activity Meter FA-st LAB.

III The pH and total titratable acidity (TTA) of dough and bread samples were determined after suspension in water by a DL22 titrator.

IV Sourdough and bread samples were fixed by freezing in liquid nitrogen, freeze-drying, and grinding using a laboratory Waring blender HGBTWTS3 (particle size <0.25 mm).

V Metabolite formation and consumption patterns for individual strains during sourdough fermentation were determined in freeze-dried sourdough samples by HPLC (sugars, organic acids) using isopropanol (50%) extraction. Free amino acids were determined by ultra HPLC using a AccQ·Tag derivatisation kit with AccQ·Fluor reagent (Publication I).

VI The volatile compounds that form during dough fermentation were analyzed using SPME-GC-MS, using a method employing multiple headspace extraction (Publication II). The volatile compound profiles found were identified by GC-MS in fresh and stale (7 day old) model rye breads (1.000 g of bread crumb), using the same GC-MS method parameters.

VII Determination of total, soluble and insoluble dietary fiber in raw materials and freeze-dried rye breads was carried out using an enzymatic/gravimetric method with an assay kit from Megazyme International Ltd. with heat-stable α-amylase, protease, and amyloglucosidase (Publication IV). Precipitated, washed, and filtered residues were dried and corrected for protein using a Kjeldahl method and for ash by treating samples in a muffle furnace at 550°C for 10 hours.

VIII The concentrations of B-complex vitamers in raw materials and freeze-dried bread samples were determined using an LC-MS isotope dilution assay method (Publication IV). The samples were extracted in an ammonium format buffer (pH 4.4) with and without an enzyme mix containing α-amylase, β-glucosidase, and acid phosphatase.

4.2.4 *Structure analysis*

I The chemical structure of amylose, amylopectin and starch (dissolved in DMSO-d6) were studied using ^{1}H, ^{13}C, and ^{31}P NMR in a Bruker Avance III 800MHz spectrometer at 313 K.

II The texture of starch gels, dough and breads was determined using a Physica MCR 301 dynamic rheometer and a TA-XT2i texture analyzer.

III The effect of baking on the properties of bread, structure of starch, and dough was studied between (I) plates in a rheometer, (II) the object plate and cover-glass in microscope, and (III) in 3 ml vials of microcalorimeter TAM III. The temperature profile (Publication V) corresponded to that employed in an industrial rye sourdough baking process.

IV For staling studies, freshly baked breads were taken out from the oven and cooled down to room temperature in a laminar flow cabinet under UV light, sealed aseptically into plastic bags, and stored at room temperature for 11 days. The samples were collected imminently after removal from the oven (0 h) and stored for 5 hours, and 11 days, the bread slices were flash cooled in liquid nitrogen, freeze dried, and ground (particle size $\approx$ 0.25 mm).

V The crystallinity of starches, flours, and freeze-dried and ground bread samples was studied using wide angle X-ray diffraction (XRD) measurements using an Ultima IV X-ray diffractometer (Publication VI).

VI ^{13}C CP-MAS NMR spectra of starch structure in starch standards, flour, freeze-dried dough and bread samples were obtained using a Bruker Avance II 600 MHz spectrometer (Publication VI).

4.2.5 *Sensory analysis*

Descriptive sensory analysis was applied to characterize the bread quality and effects of staling. The panelists (n=9) were all employees of the Competence Center of Food and Fermentation Technologies (CCFFT) and regular bread consumers. All of the panelists had completed general sensory analysis training. Descriptive attributes as well as definitions and reference materials for descriptive sensory analysis were developed and refined through group work sessions (n=2). The samples were described for selected aroma, flavor, and texture attributes that were considered important in staling dynamics. Following structure attributes – springiness, crumbliness, adhesiveness, softness, moistness as well as aroma and taste attributes such as typical bread aroma, sour, sweet, and foreign aroma were estimated. Each attribute was rated using a 15-point scale, where 0 = none and 15 = very strong.

The partial least squares regression (PLS-R) method was used to correlate the results of sensory analysis with physical parameters (moisture, pH).

The XL Stat (AddInSoft, New York, NY, USA), Unscrambler (Camo Software, Norway), Multivariate Analysis Add-in for MS Excel (Centre for Chemometrics, University of Bristol, UK) software packages were used in sensory data analysis.

5

RESULTS AND DISCUSSION

The results of this dissertation are presented and discussed in four sections. Each section presents the results of one complete study.

5.1 SOURDOUGH FERMENTATION AND BREAD QUALITY (PUBLICATION I, PUBLICATION II, PUBLICATION III)

The peculiarities of sourdough fermentation, including cyclic fermentation, contribute to the final bread quality and stability during storage.

We studied the growth of LAB and formation of metabolites and aroma compounds during the fermentation of sourdough using isothermal microcalorimetry and HPLC (Publication I) and GC-MS with multiple headspace extraction (Publication II).

5.1.1 *Determination of specific growth rates of sourdough bacteria*

The specific growth rate in sourdough is an important factor that indicates if a bacterial species has the capability to dominate in a sourdough cycle. Measuring the growth and specific growth rate of individual bacteria in nontransparent culture media such as flour suspensions and dough is technically challenging. The plating out method turned out to be too laborious and imprecise to quantify the dynamics of bacterial growth during sourdough fermentation. Calorimetric sourdough fermentation experiments were initiated and methods were developed to calculate the specific growth rate. In parallel to calorimetric measurements, substrate consumption and product formation were measured by HPLC.

In total, 11 sourdough strains (Publication I, Table 1) were isolated from 7 month old industrial sourdough, and spontaneous sourdough. Their growth in rye dough during fermentation was characterized along with two commercial starters using measurements of evolved heat, and by measuring the formation rate of sugars, organic and amino acids, and volatile metabolites. To ensure that flour bacteria did not partake in the fermentation process, the dough was prepared from 10 kGy irradiated rye flour.

The use of inoculation dough with low bacterial concentrations (10^5 – 10^6 CFU·g^{-1}) compared to those of commercial processes (10^8 - 10^9 CFU·g^{-1}), enabled us to observe both exponential growth and the maximum specific growth rates of individual strains in rye dough using measurements of evolved heat in calorimetric vials (Publication I, Table 3). When we applied backslopping at a rate ten times higher than that employed in the

industrial process, the exponential growth phase was not observed, as expected. This rate may be difficult to observe because: (I) it takes one hour before power-time curves can be recorded because of the required stabilization time, and (II) a high inoculation rate ensures that the pH is near the specific growth rate optimum.

Determination of specific growth rate using measurements of the heat evolved is confounded by the heat evolved by other processes. We found that enzymatic hydrolysis as the flour is hydrated during the first hours of sourdough fermentation is the main factor that reduces the precision of determining the specific growth rate. During incubation of a sterile blank that was not inoculated, an increase in the concentration of amino acids and sugars accompanied by heat production was observed (Publication I, Table 2). Therefore, to obtain the power-time curves of the growth of bacteria, a hydration/enzymatic hydrolysis blank was subtracted from the power-time curve.

The power-time curves of sourdough bacteria can be divided according to the shape of the curve. We observed two clusters, one having two power maximums and the other with a single maximum. All strains, with the exception of *Leuconostoc citreum*, display a single maximum power curve (Figure 4, **A** and **B**) are homofermentative LAB species. All strains that display a double maximum are heterofermentative strains (Figure 4, **C** and **D**). Chromatographic analysis reveals that the first maxima of heterofermentative strains is probably caused by the reduction of fructose into mannitol, and acetate formation.

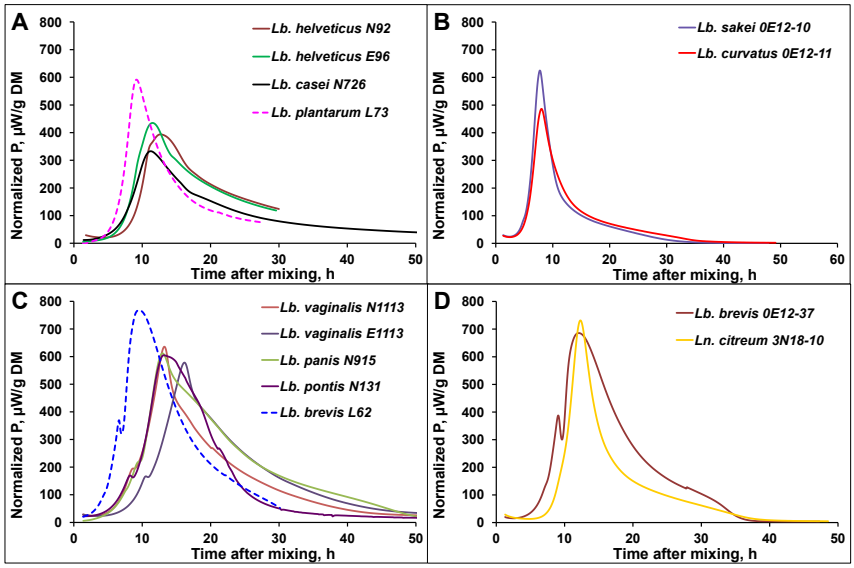


Figure 4 – Power-time curves of sourdough samples inoculated with different sourdough bacteria. **A**, **C** strains isolated from industrial sourdough and commercial strains (dashed); **B**, **D**- from spontaneously fermented laboratory sourdough. The bacteria in **A**, **B** are homofermentative and display a single maximum power curve. The bacteria in **C**, **D** are heterofermentative with most displaying two power-time maxima.

The power-time curves were used to calculate the specific maximum growth rates for different strains and species. The results, provided in Table 3 of Publication I, show that the strains with the fastest specific growth rates (*Lactobacillus curvatus*, *Lb. sakei*, *Ln. citreum*), 0.83 – 0.93 h^{-1}, were found from spontaneous sourdough that had been backslopped for 18 days (20°C, cycle time (t_{cycle}) = 24h). The strains with the slowest specific growth rates (0.45 – 0.48 h^{-1}) were found from industrial sourdough that had been backslopped for 7 months (30°C, t_{cycle} = 24h). These findings suggest that the maximum specific growth rate of strains in sourdough is a minor predictor of their stability in this industrial sourdough cycle.

We found that the main reason that strains dominate is related to the acidity and pH of the sourdough. Running in the spontaneous sourdough cycle, the initial pH after the first few backsloppings is relatively high and decreases with backslopping (cycle) number. Therefore, the strains with high growth rate but low acid resistance dominate in the first stages of sourdough cycling. In later cycles the initial pH reaches a value of 5.5 with a final pH of 3.8.

To explore the effect of pH, we produced μ-pH plots (Figure 5) by converting the power-time curves into specific growth rates (Publication I, Equations 7-11,) and to pH curves using the accumulated heat and titration curve. Sourdough used for backslopping in a ratio of 1:10 decreased the pH of the dough down to 5.5 which is lower than the optimal pH for maximum growth for all strains studied. In addition, the μ-pH plots reveal significant differences in the specific growth rate and growth behavior of LAB species in sourdough. The lactic acid bacteria isolated from spontaneous sourdough that have higher specific growth rates at the onset (*Lb. sakei* 0E12-10, *Lb. curvatus* 0E12-11, *Ln. citreum* 3N18-10) display lower specific growth rates than the "industrial" strains (*Lb. vaginalis*, *Lb. panis*, *Lb. pontis*, and *Lb. helveticus*) at pH values less than 4.5, an acidity range common within sourdoughs.

Long-term industrial sourdough cycling results in wash out of fast-growing in dough species and domination of acid-tolerant LAB populations. Some acid tolerance in long run industrial cycles was obtained at the expense of losing oxygen tolerance or perhaps was lost due to contamination of the sourdough cycle with bacteria from either the environment or flour. Interestingly, the pH tolerance and maximum specific growth rates of commercial sourdough strains belonging to the species *Lb. plantarum* and *Lb. brevis* was even higher than the industrial strains studied. It is not clear why these species did not dominate in industrial sourdoughs. No evidence was found to relate this effect to the consumption of carbon or nitrogen because the analysis shows that in all cases the glucose, maltose and all essential amino acids were present until the end of the sourdough fermentation (Publication I, Table 2; see also Section 5.1.2). It is interesting to note that *Lb. plantarum* and *Lb. brevis*, in contrast to the industrial sourdough bacteria, had high plating out yields under aerobic conditions. The results of aerobic and anaerobic fermentation in flour extract and MRS (Publication I, Table 3) suggest that oxygen is one of the most important factors determining growth performance and the formation of bacteria consortia in sourdough. In contrast to commercial freeze dried starter cultures (*Lb. plantarum* and *Lb. brevis*), the growth of industrial sourdough bacteria (*Lb. helveticus* N92, *Lb. helveticus* E96, *Lb. vaginalis* N1113, *Lb. vaginalis* E1113 and *Lb. pontis* N131) appear to be inhibited by the presence of oxygen. It is possible that the growth enhancing role of

oxygen, with regards to *Lb. plantarum* and *Lb. brevis*, is more strongly pronounced than in the oxygen phobic strains of industrial sourdough.

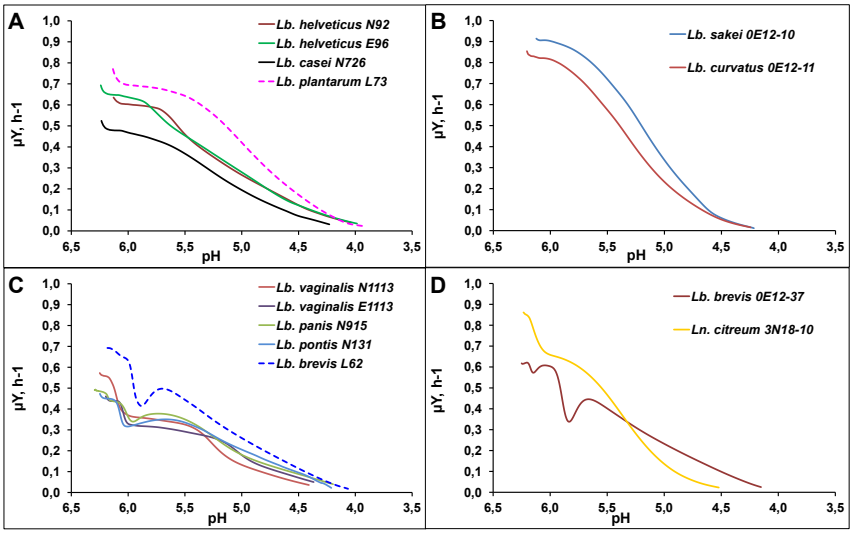


Figure 5 – Relationship between specific growth rate and pH for various LAB species. **A**, **C** strains isolated from industrial sourdough and commercial strains (dashed); **B**, **D**- from spontaneously fermented laboratory sourdough. The bacteria in **A**, **B** are homofermentative while the bacteria in **C**, **D** are heterofermentative. The curves are derived from the data presented in Figure 4. Note that plots **C** and **D** display multiple transitions.

In summary, the specific growth rate and pH tolerance are important characteristics that determine if a strain dominates in a sourdough cycle, however, they are not the only ones. The concentration of growth enhancing substrates such as oxygen, vitamers, peptides, and nucleosides *et cetera* might also be important factors that determine strain selection in a sourdough cycle.

5.1.2 *Consumption and formation of metabolites (sugars, organic acids, volatile compounds) during fermentation with individual LAB*

In addition to power–time curves, the pH, concentration of organic acids and free amino acids, sugars (Publication I, Table 2), and volatile compounds (Publication II, Table 1) were measured at different time points after dough mixing during the sourdough fermentations.

The carbon sources glucose and maltose, as well as all amino acids essential for LAB were not exhausted after 24 h of rye dough fermentation with sourdough bacteria. Therefore, the increase in acidity is the most probable reason for the decrease in both the heat flow and in the specific growth rate (Publication I, Table 3). Acetate formation occurred in heterofermentative species and was promoted by converting fructose into mannitol and its subsequent use as an electron acceptor (Publication I, Chemical Equation 1). Ex-

haustion of free fructose can be the reason for the occurrence of two maxima in the power-time curves, observed in all heterofermentative species with the exception of *Ln. citreum* (see Figure 4). Also, the aldehydes present in the dough can be used as electron acceptors as they are converted into corresponding alcohols (Publication II, Table 1) due to the reducing conditions of sourdough.

In addition to acetic acid, several volatile compounds important in rye bread flavor formation are synthesized during sourdough fermentation. Ethanol, formed by heterofermentative bacteria *Lb. brevis*, *Ln. citreum*, *Lb. vaginalis*, *Lb. panis*), reacts during sourdough fermentation with acids to form esters such as ethyl acetate and ethyl hexanoate. Acetate, in turn, reacts with alcohols forming ethyl-, hexyl- , and isopentylacetate. In contrast with heterofermentative species, homofermentative LAB species (*Lb. helveticus*, *Lb. casei*, *Lb. sakei*, *Lb. curvatus*) produce a low amount of esters. All heterofermentative species studied produce a considerable amount of 2,3-butanedione. *Lb. helveticus* sourdough strains were the only LAB studied that produced benzaldehyde.

5.1.3 *Formation of volatile compounds during baking and staling*

SPME-GC-MS volatile profiles were taken during sourdough fermentation, after bread baking, and after 7 days of storage. Laboratory rye breads were made according to the model bread recipe (Figure 3) using sourdough prepared with individual lactic acid bacteria (*Lb. helveticus* N92, *Lb. helveticus* E96, *Lb. vaginalis* N1113, *Lb. vaginalis* E1113, *Lb. brevis* L62, *Lb. casei* N726, *Lb. panis* N915, *Lb. plantarum* L73) as well as a control processed without sourdough bacteria. Most of the compounds found were common to all breads (see Table 1) and many of these are also found in sourdoughs (Publication II, Table 1). In addition to the compounds found in sourdough N,N-dimethyl-2-propanamin, 2-butenal, 1,2-dichlorethane, 2-ethylfuran, 2,4,5- trimethyl-1,3-dioxolane, 2-methoxyfuran, 3-furaldehyde, furfural, acetylfuran, and dodecane were detected in the breads. On the other hand, several compounds found in dough were not detected after baking in the breads: 2-methylpropanol, 3-methylbutanol, 2-methylbutanol, 2-pentenal, 1-penten-3-ol, 2-penten-1-ol (Z), 1-hexanol, 2-hexenal, 4-heptenal (Z), 2-heptenal (E), 2-heptanone, 2-octanone, 1-octen-3-ol, 2-nonenal, dimethyldisulfide, and dimethyltrisulfide. The inability to detect these compounds in bread can be explained by (I) a decrease in the concentration of the compounds below the detection limit due to their incorporation into the bread matrix, (II) partial vaporization, and (III) chemical modification during the baking process [113, 114].

In some cases, volatile compounds were detected in bread. Diethylacetic acid, and 1-propen-2-ol acetate were found only in bread made with *Lb. helveticus* N92, propanoic acid - in bread made with *Lb. plantarum* L73, propanal - in bread made with *Lb. vaginalis* N1113, 3-pentanone - with *Lb. vaginalis* E1113, 3-butenoic acid ethylester and 2-ethyl-2-hexenal - *Lb. helveticus* E96.

The difference in the aroma pattern of breads made by two different groups of LAB: homo- and heterofermentative bacteria were less significant than in case of respective sourdough. Regardless of this, three compounds were specific to breads made with homofermentative bacteria: 2-butenal, 2-phenyl-2-butenal, and phenylethylalcohol.

Compounds	Homofermentative strains **A**: *Lb. helveticus* N92 **B**: *Lb. helveticus* E96 **C**: *Lb. casei* N726 **D**: *Lb. plantarum* L73	Heterofermentative strains **E**: *Lb. vaginalis* N1113 **F**: *Lb. vaginalis* E1113 **G**: *Lb. panis* N915 **H**: *Lb. brevis* L62

	Blank		**A**		**B**		**C**		**D**		**E**		**F**		**G**		**H**	
	f	***s***	***f***	***s***	***f***	***s***	***f***	***s***	***f***	***s***	***f***	***s***	***f***	***s***	***f***	***s***	***f***	***s***
2-methyl propanal	+	+	+	+		+	+	+	+	+	+	+		+	+	+	+	+
1-propanol⋆					+				+		+			+	+		+	
acetic acid ethenyl ester⋆	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+
2-butanone	+	+	+		+	+					+		+	+		+	+	
N,N-dimethyl-2-propanamine⋆					+				+					+	+			
2-butenal⋆			+	+		+	+	+	+	+								
acetic acid	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1,2-dichloroethane⋆				+					+	+		+	+		+		+	
2-ethyl furan⋆	+		+	+	+	+	+	+	+		+	+	+	+	+	+	+	+
2,4,5-trimethyl-1,3-dioxolane⋆	+	+	+	+	+	+			+	+		+						
2-methoxyfuran⋆	+	+	+	+	+	+			+	+	+	+	+		+	+	+	+
2-furanmethanol⋆					+		+				+							+
isoamyl acetate⋆	+											+					+	
acetylfuran⋆		+		+		+		+		+		+		+		+		+
α-pinene						+						+		+				
acetic acid hexyl ester⋆	+	+										+	+		+	+	+	+
phenylethylalcohol⋆				+		+				+								
2-octenal	+		+		+										+			
heptanoic acid ethyl⋆	+	+									+	+					+	+
nonanal	+	+	+		+						+	+	+	+	+	+	+	+
dodecane⋆		+		+		+				+				+		+		+
carvone⋆																	+	+
2-phenyl-2-butenal⋆	+	+	+	+	+	+	+	+		+								

Table 1 – Volatile compounds profile in fresh (0h, ***f***) and staled (7d, ***s***) model rye breads made with sourdough prepared with different LAB. The presence of a compound is marked by "+". Compounds found only in breads are labeled with ⋆. The following compounds were detected under all conditions: ethyl acetate, 3-methyl butanal, 2-methyl butanal, L-Leucine methyl ester, 1-pentanol, hexanal, 3-furaldehyde⋆, furfural⋆, styrene⋆, heptanal⋆, benzaldehyde, 2-pentylfuran, hexanoic acid ethyl ester, benzacetaldehyde⋆, octanoic acid ethyl ester⋆.

During staling, only a few changes in the aroma profiles were observed. The comparison of aroma profiles of fresh bread and 7 day-old breads revealed that 2-acetylfuran was released from the bread matrix in an amount detectable by SPME-GC-MS only after staling. 2-acetylfuran has almonds, nuts, fermented aroma, milk, and sweet caramel-like aroma [115] and is known to be a compound formed during baking as one of important compound of the Maillard reaction [116, 117]. The other two compounds which apperar in the volatile pattern during staling were α-pinene and phenylethylalcohol. The first has a dry woody odor characterized as a component of fruity juice and some ether oils [118], and phenylethylalcohol has a flowery odour and is considered a favorable bread flavor [119]. How the volatiles 2-acetylfuran, α-pinene, and phenylethylalcohol are related to the process of developing an "old bread" aroma is currently unknown, but could be studied using GC-MS/olfactometry.

The aroma patterns of the industrial breads determined by SPME-GC-MS/olfactometry were richer than those determined for model breads using individual strains. In addition to the compounds found in the model experiments using SPME-GC-MS a number of other compounds were identified using olfactometry: 2-propanone (acetone), 2-butanone (ether), 2,3-butanedoine (butter), formic acid methyl ester, 1-hydroxy-2-propanone, 1-penten-3-one (fish, pungent), 1-hexanol (resin, flower), heptanoic acid (fatty, frutty), 2,3-pentanedione and 3-hydroxy-2-butanone (cream, butter), ethyllactate (fruit), and acetylpyrrole (nut, bread, walnut). It is probable, that most of those compounds were also present in the laboratory sourdoughs but remained below the detection limit of the method used to study the effect of staling on aroma formation.

5.2 STABILITY OF DIETARY FIBER AND VITAMERS DURING RYE BREAD PROCESSING (PUBLICATION IV)

Due to the complexity of the analytical procedures for determining dietary fiber and vitamins, the recipe based calculation of nutrient content in breads has become common practice in the baking industry. However, sourdough fermentation, leavening with yeast, and baking can all induce chemical changes that make the results of the calculations erroneous.

5.2.1 *Stability of dietary fiber during rye sourdough bread processing*

The content of indigestible material might change during bread processing, especially during fermentation and baking. The effect of processing was studied by determining the content of soluble and insoluble dietary fiber using the enzymatic-gravimetric method in both the raw materials and breads prepared from these raw materials.

The results (Publication IV, Table 2), demonstrate that the content of total dietary fiber during processing increases. An increase in total dietary fiber during bread processing has been observed previously. Johansson and coauthors [120] observed an increase in total dietary fiber in wheat and rye breads made from low extraction flour and attributed this to the formation of starch resistant to digestion during baking.

The ratio of soluble to insoluble dietary fiber increased during rye bread processing. The measured concentration of soluble fiber was 3.4 and 2.6% of the dry matter (DM) in rye bread and fine rye bread, respectively. These measurements are significantly higher than the recipe based values (2.0% and 1.5% DM, respectively). The increase in soluble dietary fiber content during bread processing can be explained by the partial conversion of insoluble fiber into the soluble variety during rye flour fermentation, scalding, leavening with yeast, and during the first stages of baking. Such a redistribution of dietary fiber forms by the activities of intrinsic enzymes in rye flour (α–amylase, β–xylosidase, α–arabinofuranosidase, β-glucanase, endo-xylanase and cinnamoyl esterase) was suggested by Boskov Hansen and coauthors [62]. The enzymatic hydrolysis of non-soluble arabinoxylan might improve the gas holding capacity of dough [80]; however further hydrolysis of soluble arabinoxylan can decrease it. On the other hand, low pH values of sourdough may inhibit this process. The tight correlation of this process with sensory properties (provided in Figure 6) has yet to be demonstrated.

5.2.2 *B-complex vitamers in cereal materials and rye bread*

Many forms of processing, including sourdough fermentation, change the concentration of vitamins in baked bread. In this study we measured the content of vitamers (thiamine, riboflavin, nicotinic acid, nicotinamide, pyridoxine, pyridoxal, pantothenic acid) in both raw materials (rye flours, white and red rye malt, yeast) and in rye sourdough breads produced from these raw materials. A method was developed that made use of LC-MS with a stable isotope dilution assay combined with enzymatic treatment.

Enzymatic hydrolysis was carried out in combination with the process of extracting the B-complex vitamers from the complex sample matrices using acid phosphatase, β-glucosidase, and α-amylase. The use of these enzymes increased the concentration of thiamine (10 - 55%), riboflavin (10 - 35%), nicotinamide (25 - 60%), and pyridoxal (40%) due to conversion of respective cofactors (TTP, NADH, NADPH, NAD+, NADP+, PLP, CoA, FMN, FAD) into the vitamin forms listed above (Publication IV, Table 3).

The content of B-complex vitamers in rye bread was calculated on the basis of the recipes used to make rye breads (supplemented with our improved measurements of the vitamer concentrations in the raw materials). In almost all cases, the recipe based estimation of vitamer content was higher than those determined experimentally in the finished product: thiamine 60%, riboflavin 20%, pyridoxal 150%, pyridoxine 60%, and pantothenic acid 10%. The loss of thiamine, riboflavin and pyridoxine during thermal treatment (baking) was similar to that reported earlier for breads [36, 43, 44].

In contrast to all other vitamers, the concentration of nicotinamide increased during processing by ten times. To explore this effect we compared the 10 kGy irradiated control with a sample that underwent fermentation. In the control, the nicotinamide concentration was constant during incubation (around 10 $\mu g \cdot 100 g_{DM}^{-1}$). In contrast, the concentration of nicotinamide increased to 600 $\mu g \cdot 100 g_{DM}^{-1}$ in the sample that underwent fermentation. In the same fermentation, nicotinic acid decreased from 500 to 80 $\mu g \cdot 100 g_{DM}^{-1}$ (Publication IV, Table 3). The effect can be explained by the conversion of

nicotinic acid and/or NAD+ into nicotinamide by *Lactobacilli* over a series of enzymatic reactions [121].

Interestingly, the concentration of pantothenic acid increased during the incubation of flour from 400 to 550 $\mu g \cdot 100 g_{DM}^{-1}$ yet during sourdough fermentation it decreased from 400 to 225 $\mu g \cdot 100 g_{DM}^{-1}$ (Publication IV, Table 3). Such drastic changes in concentration were not observed during lactic acid fermentation for the other vitamers studied.

5.3 STABILITY OF SENSORY PROPERTIES

5.3.1 *Effect of sourdough fermentation*

We studied the effect of isolated bacterial species on the sensory properties of rye bread. Rye breads were produced according to the model recipe (Figure 3). Sourdough breads were prepared using isolated individual LAB or mixed suspensions of *Lb. helveticus* N92 + *Lb. vaginalis* N1113, or *Lb. plantarum* L73 + *Lb. brevis* L62, or *Lb. helveticus* N92 + *Lb. panis* N915. Rye model bread made with *Lb. helveticus* showed high springiness. Porosity, adhesiveness, and sweetness are terms that best describe bread made using *Lb. panis* and *Lb. brevis* + *Lb. plantarum*. We correlated the most important sensory parameters that characterize the staling of model breads such as moistness, springiness, typical aroma, typical taste, crumbliness, porosity, sweetness, "old bread" taste (foreign or off-taste) with measured parameters (pH, TTA, water content). The correlation shows that a cluster of bread made without sourdough (Blank) or with *Lb. vaginalis* or *Lb. casei* strains had poor sensory characteristics and are thus situated at the lower right corner of the PLS-R plot (Figure 6, dashed).

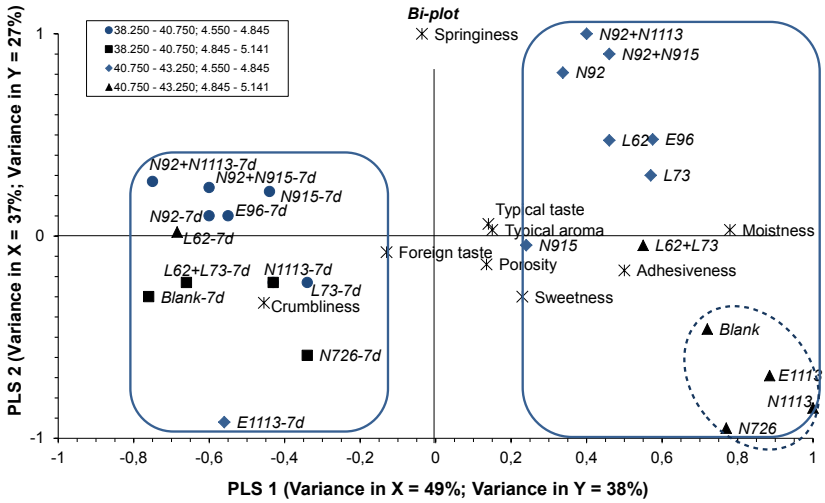


Figure 6 – Fresh and 7 day-old breads (labeled "7d") sorted by pH and moisture using the PLS-R method. The signs of samples correspond to moisture content (38-43%) and pH (4.5-5.1). N92 is *Lb. helveticus* N92; E96 is *Lb. helveticus* E96; N1113 is *Lb. vaginalis* N1113; E1113 is *Lb. vaginalis* E113; L73 is *Lb. plantarum* L73; L62 is *Lb. brevis* L62; N915 is *Lb. panis* N915; N726 is *Lb. casei* N726; N92+N915 is *Lb. helveticus* N92 + *Lb. panis* N915; N92+N1113 is *Lb. helveticus* N92 + *Lb. vaginalis* N1113; L62+L73 is *Lb. brevis* L62 + *Lb. plantarum* L73.

The results of sensory analysis grouped according to pH show that breads in the upper part of the graph are sourer and had a lower pH than those at the bottom. A good correlation between pH and springiness was observed. According to Arendt *et al.* [2] the correlation between acidity and the structure of bread can be explained by the ability of rye pentosans to bind water and enhance the water-binding of starch due to the inhibition of enzymatic activity under acidic conditions.

Fresh breads that have desirable properties such as sweetness, moistness, porosity, adhesiveness, crumbliness, and a typical rye bread flavor profile, are positioned at the rightmost part of graph (Figure 6). After staling, breads clustered into a more compact group with comparable properties ("old bread" cluster). This clustering was independent of the starter strains used.

These results suggest that our sourdough process, and probably most sourdough processes, have a limited influence on rye bread staling. The choice of strain used to carry out the sourdough fermentation does, however, have a significant effect on bread quality.

5.3.2 *Comparison of sensory properties of wheat and rye breads during staling*

Three different rye sourdough and wheat breads were purchased from a local market to characterize, by sensory analysis, the aging of breads during the 3 days before and 2 days after the shelf life stated by the producer. The results show that products belonging to

the rye sourdough cluster stale less significantly than those of the wheat bread cluster (Figure 7). A change was observed in many important quality indicators during bread storage (springiness, softness, odor and flavour intensity, and moistness). These changes are more pronounced and significant in wheat breads compared with rye breads.

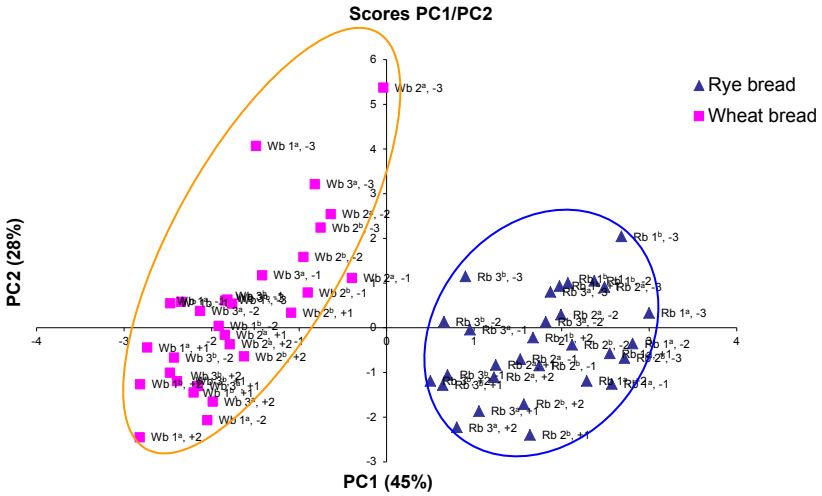


Figure 7 – Principal component analysis (PCA) of the sensory properties of breads during storage. Rb1, Rb2, Rb3 – rye sourdough breads, Wb1, Wb2, Wb3 – wheat breads, -3, -2, -1, +1, +2 indicates the age of bread in days relative to the expiry date. The breads in parallel sessions are labeled with superscripts [a] and [b].

Variation of parameters was higher in the case of wheat bread during storage, particularly during first days. The changes in sensory parameters of rye breads were less significant. Also the rate of decrease of most important parameters like aroma, taste, softness, and moistness was slower in rye breads than in wheat breads. The differences can be related to low pH and high amount of dietary fiber as well as lower porosity of rye breads.

5.4 STRUCTURAL CHANGES OF RYE BREAD DURING STALING (PUBLICATION V , PUBLICATION VI)

The results of our sensory analysis reported in the previous section reveal that wheat breads stale more significantly than those of rye. We followed up on this work by determining to what extent differences in starch structure and behavior during baking and storage could affect the staling rate in wheat and rye breads.

5.4.1 *Chemical structure of starch and content in dough and breads*

The structure of rye and wheat starch was characterized by ^{13}C NMR (Publication VI). The spectra did not show significant differences in the chemical structure or content of

amylopectin between wheat and rye starch, and the amylopectin in both were found to be similar to that of maize amylopectin. A highly ordered structure of amylopectin has been proposed (Publication VI, Figure 5). The branching region in both rye and wheat amylopectin takes 5 × 10 = 50% of the glucose units, and a ratio of about 2.5 medium glucose cycle chains per ten branching glucose cycles was calculated (Publication VI). While the chemical structure of rye and wheat amylopectin and amylose was very similar, significant differences are observed in the starch phospholipid fraction using ^{31}P NMR (Publication VI, Figure 6).

The amount of starch in wheat and rye breads was different; 71 g in wheat and 63 g per 100 g of dry matter in rye breads (Table 2). This difference can affect bread staling and starch retrogradation more than the small differences observed in the starch chemical composition.

Table 2 – Starch and water content in flours and breads.

Sample	Total starch $g{\cdot}100g^{-1}_{DM}$	Water content $g{\cdot}100g^{-1}_{sample}$
Wheat flour (W550)	76.11 ± 1.01	12.6 ± 0.2
Rye flour, wholemeal	61.23 ± 1.39	11.3 ± 0.2
Rye flour, dark	65.96 ± 1.77	11.4 ± 0.2
Wheat dough	67.35 ± 0.64	41.4 ± 0.5
Wheat bread	71.20 ± 4.51	39.8 ± 0.7
Rye dough	64.15 ± 5.43	43.6 ± 0.8
Rye bread	63.30 ± 5.02	42.4 ± 0.5

5.4.2 *Study of starch and dough characteristics using microbaking*

To study the effect of baking on the structural properties of dough and starch, a temperature profile was simulated on microscope slides, calorimetric vials, and between rheometer plates. The water concentration of each specimen was set to that measured in the rye dough (Table 2) and the temperature profile to that measured in the middle of the crumb in the industrial process (Publication V, Publication VI).

During microscopic observation, a loss of birefringence was observed in starch and dough samples (indicated by a losses of the typical Maltese cross pattern) after baking indicated the disordering of radial arrangement of starch molecules in the granule (Publication VI, Figure 1). The appearance and increase of intensity of longish structures were observed in all starch and bread samples during cooling and storage. Those longish structures were not found in amylopectin samples and may be related both to the leakage of amylose from starch granules and the crystallization of amylose [122]. Only in the case of a pure amylopectin preparation, structures characterized by a Maltese cross reappeared during storage. No visual differences were observed between rye and wheat starch nor their respective dough during micro-baking on microscopic slides.

However, significant differences in the behavior of rye and wheat starch and dough were observed during microbaking in both the microcalorimeter and rheometer (Publication V, Publication VI).

The storage modulus, G′, profiles of rye and wheat starch and dough micro-baking were different. The storage modulus of rye starch started to increase at a higher temperature than that of wheat starch (45°C and 40°C, respectively) (Publication V, Figure 2). Further increase in temperature decreased G′ due to liquidation of the starch gel. An increase in G′ within dough samples was observed at a higher temperature (around 65°C). This increase could be related to the effects of protein denaturation in the dough matrices. A further decrease in G′ in both doughs at 20°C may be explained by continuous mechanical stress that alters the bread structure.

The thermal activity of micro-baked rye and wheat dough samples and starch suspensions was measured (Publication V, Figure 3A). This work demonstrates that the heat flow (μW per gram of starch in sample) during the period of 2-48 h after baking is lower in rye starch and rye dough compared to wheat starch and bread, respectively.

This might be related to different rates or intensities of phase transition processes (crystallization and glass transition) in rye and wheat dough after baking. Silverio and coauthors [123] suggest that the heat flow during the first 5 h to 10 h is related to amylose crystallization, which is in good agreement with our microscopic observation that longish structures form within the first hours after baking.

5.4.3 *Study of starch and bread structure using X-ray diffraction*

To study the changes in the crystalline structures of starch and flours during baking, cooling and storage X-ray diffraction spectra of starches and freeze-dried breads were measured. Rye and wheat starch spectra (Figure 8A) were very similar to that of amylopectin which suggests that the amylose content and phospholipid composition have a small effect on the crystalline structure of starch. The X-ray diffraction pattern of corn and potato starch were different, however.

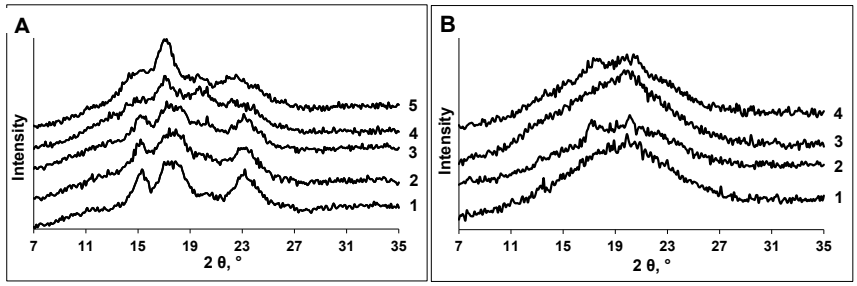


Figure 8 – XRD spectra of amylopectin and starches. Subplot **A**: maize amylopectin (1), starches from rye (2), wheat (3), corn (4), potato (5) and breads. Subplot **B**: rye bread, 0 h (1), rye bread, 11 days (2), wheat bread, 0 h (3), wheat bread, 11 days (4).

The spectra of rye and wheat bread after baking and after 11 days of staling are similar (Figure 8B). We observed that the crystalline patterns found in staled bread resemble B-type potato starch. From XRD data we can conclude that during starch retrogradation part of the water is removed from the bread matrix into the crystalline structures of starch. Supposing that after gelatinization of starch during baking the amylose and amylopectin starts to crystallize into hydrated forms (for example Vh or B-type) that contain about 30% water [99] and about 50% starch, a considerable amount of water (15 g per 100 g of bread) can be removed from the crumb amorphous phase into the crystalline starch structures during bread storage. Binding water from amorphous bread structures into crystalline structures can explain the dry mouth-feel that appears in aged breads. According to our XRD analysis, we find an increase in the degree of crystallization of starch from 3 to 21% for rye sourdough bread and from 3% to 26% for wheat bread during 11 days of storage (Publication VI, Table 1). This corresponds to an amount of water binding of about 3 g per 100 g of bread, which all together makes up about 7.5% of the water. This is a considerable decrease in water concentration which can cause both a dry mouth feel and an increase in bread firmness during storage.

To study the effect of different LAB strains (homofermentative and heterofermentative) on the crystallinity of staled breads, rye model breads made with *Lb. plantarum* and *Lb. brevis* were compared by XRD. No difference in relative crystallinity of rye breads made using different LAB strains was observed during storage (Publication VI, Figure 3). So the effect of sourdough on bread structural properties is not related to starch retrogradation, but related rather to decreasing the pH and affecting hydrolytic processes and water holding capacity.

5.4.4 ^{13}C CP-MAS NMR spectra of rye and wheat breads during staling

The recrystallization of starch in hydrated forms was supported by ^{13}C CP-MAS NMR spectra of rye and wheat breads before and after staling (Figure 9).

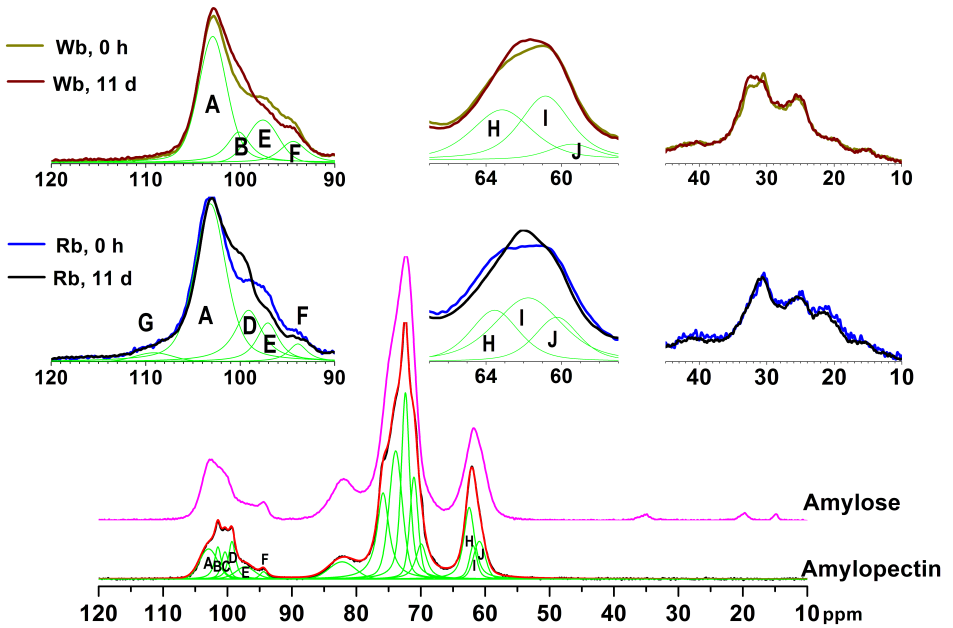


Figure 9 – ^{13}C CP-MAS NMR spectra of amylopectin, rye and wheat breads. Rb – rye bread, Wb – wheat bread.

Interpretations of the individual components: C1-A (102.9-103.2 ppm) – amorphous region of amylopectin (branching points) and V-type single helix; C1-B (101.4-101.5 ppm) – double helices; C1-C (100.1-100.5 ppm) – double helices; C1-D (99.1-99.8 ppm) – double helices; C1-E (96.4 – 97.7 ppm) – glucose units near α-(1→6) linkages within the branched regions; C1-F (93.8 – 94.7 ppm) – associated with constrained linkages; C1-G – 106.9-109.1 ppm- C1 of rye cellulose and mixed-linkage (1→3)(1→4)-β-D-glucans.

The peak A (103 ppm) in the C1 region is typical for the V-type single helix and dominates in all NMR MAS spectra. Its relative intensity did not change during bread baking. The decrease in signal intensity in the region related to the crystalline structure (102-99 ppm, peaks B, C and D) and an increase in relative intensity of the broad peak in the range of 98 - 96 ppm, which corresponds to a non-crystalline region, were observed after baking. During staling, the area of this region decreased and that of the crystalline region (101-99 ppm) increased. The MAS NMR spectra demonstrate that starch in rye sourdough breads crystallize in somewhat different forms than in wheat bread (region C1 and C6 on the Figure 9).

During staling of rye bread, the appearance of a peak with maximums at 99.3 ppm was observed. A similar peak appeared in wheat bread at 100.1 ppm. This suggests that these breads display differences in starch retrogradation. Additionally, peak at 106.1 – 109 ppm (C1 – G) are observed only in rye flour and bread, and not in starch. This

is probably explained by the presence of mixed-linkage (1→3)(1→4)-β-D-glucans with cellulose-like fragments in rye dark flour [66, 124–126].

In addition, within C-4 some changes during baking were observed. However, like those of C2, C3, C5 region, these could not be related to structural changes. NMR spectra show changes during baking and storage also in the C6 region. The maximum shifted from 62 to 61 ppm and restored in rye and wheat bread in slightly different forms (Figure 9). In the protein/lipid region 35-10 ppm differences in wheat and rye spectra were quite significant. The differences can be related to transformations within the gluten structure and/or formations of starch gluten complexes in the wheat breads.

Also, a different amount of water can cause the different rates of staling of rye and wheat bread. The recrystallisation of starch amylopectin and amylose in a hydrated form causes the decrease in availability of water during storage and the development of a dry mouth feel during consumption. Taking into account the higher water and lower starch content in rye bread compared to wheat bread (Table 2), the relative amount of water which can be bound into the crystalline structures of starch in wheat bread during retrogradation is larger. This, in turn, can explain the lower effect of starch crystallization on rye bread quality in comparison to wheat bread.

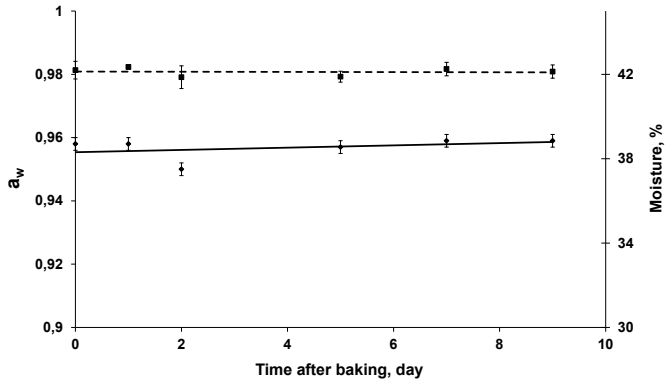


Figure 10 – Water activity (a_w) and moisture in rye bread crumb after baking (firm line – water activity, dotted line – moisture).

The decrease in water activity expected in the case of co-crystallization into chemically bound forms with amylopectin was not observed in this study (Figure 10). This might be explained by the effect of removing water on bread water activity in the non-crystalline phase and/or parallel processes like glass transition occurring during staling. Incorporation of water from the bread crumb into crystalline structures of starch during bread storage will also increase the concentration of soluble chemicals in bread. This can initiate transition from a rubber state into a glass state which might explain the increase in firmness and storage module during bread staling.

SUMMARY

6

CONCLUSIONS

The main goal of this dissertation is to evaluate the factors that influence both the nutritional makeup and sensory quality of rye sourdough bread during processing and storage. The results of the study are summarized as follows:

I A laboratory rye sourdough process was developed, including a method for characterization of individual lactic acid bacteria during sourdough fermentation using isothermal microcalorimetry.

II *Lactobacillus helveticus* was found to be present during all stages of propagation and was the dominant strain in industrial rye sourdough. A number of dominant lactic acid bacteria strains isolated from rye industrial and spontaneously fermented laboratory rye sourdough were characterized by their heat production and metabolite production/consumption during sourdough fermentation.

III Oxygen availability and acidity are the most important factors that determine the formation and stability of bacteria consortia in a sourdough cycle. The LAB strains that dominate in the industrial sourdough cycle had better acid tolerance but lower maximum growth rate and oxygen tolerance than species isolated from a spontaneously started sourdough cycle.

IV Significant differences are observed in the volatile profiles of dough fermented with different bacteria; However, these differences are equalized to a great extent during baking.

V B-complex vitamins, with exception of B3, are mostly present in "simple" forms in bread, and are partly degraded during processing. The content of dietary fiber and vitamers in the ingredients used to make the bread and the finished product can differ significantly and caution should be taken when calculating the nutrient content of bread on the basis of the ingredients used to make it.

VI The ratio of soluble to insoluble dietary fiber increased during rye sourdough processing by the activity of both endogenous flour enzymes and lactic acid bacteria enzymes during fermentation. This not only affects the nutritional content but also the sensory properties of rye bread.

VII The XRD, ^{13}C NMR, ^{1}H NMR, ^{13}C CP-MAS NMR spectra reveal that the structure of the carbohydrate backbone of rye and wheat starch are very similar. The rye and wheat amylopectin has a very similar and highly ordered chemical structure. Differences were found in the phospholipid structure by ^{31}P NMR.

VIII The effect of sourdough on bread structural properties is not related to starch retrogradation, but related rather to decreasing the pH and affecting hydrolytic processes and water holding capacity.

IX After baking, starch recrystallization occurs with water incorporation into the crystalline structure of starch forming B-type and V-type starch crystallites and may be the main reason for bread staling. Different proportions of water and changes in starch fine structure cause the different rates of staling of rye and wheat bread.

BIBLIOGRAPHY

BIBLIOGRAPHY

[1] United Nations. FAOStat: Statistical database of food and agriculture organization of the United Nations. http://faostat.fao.org/, 2012.

[2] E. Arendt, L. Ryan, and F. Dal Bello. Impact of sourdough on the texture of bread. *Food Microbiol.*, 24(2):165–174, 2007.

[3] B. J. Wood. *Microbiology of Fermented Foods, Volumes 1 and 2.* Springer, 1997.

[4] W. Seibel. The sourdough process for bread in germany. *Cereal foods world*, 36(3):299–304, 1991.

[5] G. Oettler. The fortune of a botanical curiosity – Triticale: Past, present and future. *J Agric Sci*, 143(05):329–346, 2005.

[6] B. Oliete, G. Pérez, M. Gómez, P. Ribotta, M. Moiraghi, and A. León. Use of wheat, triticale and rye flours in layer cake production. *Int J Food Sci Technol*, 45(4):697–706, 2010.

[7] L. Ask, B. Nair, and N. Asp. Effect of scalding procedures on the degradation of starch in rye products. *J. Cereal Sci.*, 13(1):15–26, 1991.

[8] C. J. A. M. Keetels, G. T. Oostergetel, and T. Van Vliet. Recrystallization of amylopectin in concentrated starch gels. *Carbohydr Polym*, 30(1):61–64, 1996.

[9] M. Häggman and H. Salovaara. Microbial re-inoculation reveals differences in the leavening power of sourdough yeast strains. *Lebensm. Wiss. Technol.*, 41(1):148–154, 2008.

[10] L. De Vuyst and P. Neysens. The sourdough microflora: Biodiversity and metabolic interactions. *Trends Food Sci. Technol.*, 16(1-3):43–56, 2005.

[11] K. Katina, M. Sauri, H. . Alakomi, and T. Mattila-Sandholm. Potential of lactic acid bacteria to inhibit rope spoilage in wheat sourdough bread. *Lebensm. Wiss. Technol.*, 35(1):38–45, 2002.

[12] W. Hammes, M. Brandt, K. Francis, J. Rosenheim, M. H. Seitter, and S. Vogelmann. Microbial ecology of cereal fermentations. *Trends Food Sci. Technol.*, 16(1-3):4–11, 2005.

[13] L. De Vuyst and M. Vancanneyt. Biodiversity and identification of sourdough lactic acid bacteria. *Food Microbiol.*, 24(2):120–127, 2007.

[14] P. Decock and S. Cappelle. Bread technology and sourdough technology. *Trends Food Sci. Technol.*, 16(1-3):113–120, 2005.

[15] M. A. Müller, G. Wolfrum, P. Stolz, M. Ehrmann, and R. Vogel. Monitoring the growth of *Lactobacillus* species during a rye flour fermentation. *Food Microbiol.*, 18(2):217–227, 2001.

[16] K. Kulp and K. Lorenz. Sourdough bread in finland and eastern europe. In T. Valjakka, H. Kerojoki, and K. Katina, editors, *Handbook of Dough Fermentations*. Marcel Dekker Inc., 2003.

[17] H. Salovaara. Lactic acid bacteria in cereal-based products. In S. Salminen, A. von Wright, and A. Ouwehand, editors, *Lactic Acid Bacteria*, pages 431–452. CRC Press, Marcel Decker Inc, 1st edition, 2004.

[18] W. Hammes and R. Vogel. The genus of lactobacillus. In W. Holzapfel and B. Wood, editors, *The Genera of Lactic Acid Bacteria*. Blackie Academic & Professional, 1995.

[19] M.G. Gänzle, M. Ehmann, and W.P. Hammes. Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of sourdough fermentation. *Appl. Environ. Microbiol.*, 64(7):2616–2623, 1998.

[20] A.S. Hansen. Sourdough bread. In Y. Hui, L. Meunier-Goddik, Ö. Solvejg Hansen, J. Josephsen, W. Nip, P. Stanfield, and F. Toldra, editors, *Handbook of Food and Beverage Fermentation Technology*. Marcel Dekker Inc., 2004.

[21] H. Rosenquist and Å. Hansen. The microbial stability of two bakery sourdoughs made from conventionally and organically grown rye. *Food Microbiol.*, 21(3):241–250, 2000.

[22] L. De Vuyst, G. Vrancken, F. Ravyts, T. Rimaux, and S. Weckx. Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota. *Food Microbiol.*, 26(7):666–675, 2009.

[23] C. Meroth, W. Hammes, and C. Hertel. Identification and population dynamics of yeasts in sourdough fermentation processes by PCR-denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.*, 69(12):7453–7461, 2003.

[24] S. Siragusa, R. Di Cagno, D. Ercolini, F. Minervini, M. Gobbetti, and M. De Angelis. Taxonomic structure and monitoring of the dominant population of lactic acid bacteria during wheat flour sourdough Type I propagation using *Lactobacillus sanfranciscensis* starters. *Appl. Environ. Microbiol.*, 75(4):1099–1109, 2009.

[25] S. Abd-Aziz, G. Hung, M. Hassan, M. Abdul Karim, and N. Samat. Indirect method for quantification of cell biomass during solid-state fermentation of palm kernel cake based on protein content. *Asian J. Sci. Res.*, 1(4):385–393, 2008.

[26] M. R. Terebiznik and A. M. R. Pilosof. Biomass estimation in solid state fermentation by modeling dry matter weight loss. *Biotechnology Techniques*, 13(3):215–219, 1999.

[27] L. Gustafsson. Microbiological calorimetry. *Thermochim Acta*, 193:145–171, 1991.

[28] L. Wadsö and F. Gómez Galindo. Isothermal calorimetry for biological applications in food science and technology. *Food Control*, 20(10):956–961, 2009.

[29] N. Kabanova, A. Kazarjan, I. Stulova, and R. Vilu. Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at different glucose concentrations in broth. *Thermochim Acta*, 496(1-2):87–92, 2009.

[30] A. Menert, M. Liiders, T. Kurissoo, and R. Vilu. Microcalorimetric monitoring of anaerobic digestion processes. *J Therm Anal Calorim*, 64(1):281–291, 2001.

[31] A. Dobson, O. O'sullivan, P. Cotter, P. Ross, and C. Hill. High-throughput sequence-based analysis of the bacterial composition of kefir and an associated kefir grain. *FEMS Microbiol. Lett.*, 320(1):56–62, 2011.

[32] Finish National Institute for Health and Welfare, Nutrition Unit. Finish food composition database FINELI, Release 11. `http://www.fineli.fi/`, 2012.

[33] Department of Nutrition, National Food Institute, Technical University of Denmark. Danish food composition data, revision 7.01. `http://www.foodcomp.dk`, 2012.

[34] Department of Agriculture, Agriculture research Centre. United States department of agriculture (USDA) national nutrient database. `http://ndb.nal.usda.gov/`, 2012.

[35] A. Michalska, A. Ceglinska, R. Amarowicz, M. Piskula, D. Szawara-Nowak, and H. Zielinski. Antioxidant contents and antioxidative properties of traditional rye breads. *J. Agric. Food Chem.*, 55(3):734–740, 2007.

[36] K. Katina, A. Laitila, R. Juvonen, K. Liukkonen, S. Kariluoto, V. Piironen, R. Landberg, P. Aman, and K. Poutanen. Bran fermentation as a means to enhance technological properties and bioactivity of rye. *Food Microbiol.*, 24(2):175–186, 2007.

[37] A. Rakha, P. Åman, and R. Andersson. Characterisation of dietary fibre components in rye products. *Food Chem*, 119(3):859–867, 2010.

[38] C. Martinez-Villaluenga, A. Michalska, J. Frias, M. Piskula, C. Vidal-Valverde, and H. Zieliński. Effect of flour extraction rate and baking on thiamine and riboflavin content and antioxidant capacity of traditional rye bread. *J. Food Sci.*, 74(1):C49–55, 2009.

[39] P. Finglas. Thiamin. *Int J Vitam Nutr Res*, 63(4):270–274, 1993.

[40] G. Rindi and U. Laforenza. Thiamine intestinal transport and related issues: Recent aspects. *Proc. Soc. Exp. Biol. Med.*, 224(4):246–255, 2000.

[41] M. Buchholz, A. Drotleff, and W. Ternes. Thiamin (vitamin B1) and thiamin phosphate esters in five cereal grains during maturation. *J. Cereal Sci.*, 56(1):109–114, 2012.

[42] L. Ye, R. Eitenmiller, and W. Landen. *Vitamin Analysis for the Health and Food Sciences*. CRC Press, 2nd edition, 2008.

[43] F. Batifoulier, M. . Verny, E. Chanliaud, C. Rémésy, and C. Demigné. Effect of different breadmaking methods on thiamine, riboflavin and pyridoxine contents of wheat bread. *J. Cereal Sci.*, 42(1):101–108, 2005.

[44] F. Batifoulier, M. . Verny, E. Chanliaud, C. Rémésy, and C. Demigné. Variability of b vitamin concentrations in wheat grain, milling fractions and bread products. *Eur J Agron*, 25(2):163–169, 2006.

[45] P. Ottaway. The stability of vitamins during food processing. In C. Henry and C. Chapman, editors, *The Nutrition Handbook for Food Processors*. Woodhead Publishing in Food Science and Technology, 2002.

[46] Y. Suzuki and K. Uchida. Biosynthesis of riboflavin-alpha-glucoside by plant grains. *Arch. Biochem. Biophys.*, 130(1):683–684, 1969.

[47] G. Ball. *Vitamins In Foods*. CRC Tailor and Francis Group, 2006.

[48] H. Ghosh, P. Sarkar, and B. Guha. Distribution of the bound form of nictotinic acid in natural materials. *J. Nutr.*, 79:451–453, 1963.

[49] J. Wall and K. Carpenter. Variation in availability of niacin in grain products. *Food Technol*, 42(10):NA, 1988.

[50] M. Kall. Determination of total vitamin B6 in foods by isocratic HPLC: A comparison with microbiological analysis. *Food Chem*, 82(2):315–327, 2003.

[51] J.E. Leklem. Vitamin B6. In R.B. Ruker and J. Suttie, editors, *The Handbook of Vitamins*, pages 339–393. Marcel Dekker, Inc., 3rd edition, 2001.

[52] World Health Organisation. Vitamin and mineral requirements in human nutrition. joint FAO/WHO expert consultation on human vitamin and mineral requirements. `http://www.who.int/nutrition/publications/micronutrients/9241546123/en/index.html`, 2012.

[53] E. Gujska, J. Michalak, and J. Klepacka. Folates stability in two types of rye breads during processing and frozen storage. *Plant Foods Hum Nutr*, 64(2):129–134, 2009.

[54] S. Kariluoto, L. Vahteristo, H. Salovaara, K. Katina, K. Liukkonen, and V. Piironen. Effect of baking method and fermentation on folate content of rye and wheat breads. *Cereal Chem*, 81(1):134–139, 2004.

[55] K. E. Bach Knudsen. The nutritional significance of "dietary fibre" analysis. *Anim. Feed Sci. Technol.*, 90(1-2):3–20, 2001.

[56] J. Slavin, D. Jacobs, L. Marquart, and K. Wiemer. The role of whole grains in disease prevention. *J Am Diet Assoc*, 101(7):780–785, 2001.

[57] J. Mann and J. Cummings. Possible implications for health of the different definitions of dietary fibre. *Nutr Metab Cardiovasc Dis*, 19(3):226–229, 2009.

[58] J.M. Jones. Dietary fibre or whole grains or both? In Hannu Salovaara, Fred Gates, and Maija Tenkanen, editors, *Dietary Fibre Components And Functions*, pages 13–31. Wageningen Academic Pub, 2007.

[59] S. C. Lee, L. Prosky, and J. W. De Vries. Determination of total, soluble, and insoluble dietary fiber in foods–enzymatic-gravimetric method, MES-TRIS buffer: Collaborative study. *J. Assoc. Official Agric. Chem.*, 75(3):395–416, 1992.

[60] L. Prosky, N. Asp, T. Schweizer, J. Devries, and I. Furda. Determination of insoluble, soluble, and total dietary fiber in foods and food products: Interlaboratory study. *J Assoc Off Anal Chem*, 71(5):1017–1023, 1988.

[61] O. Theander, P. Aman, E. Westerlund, R. Andersson, and D. Pettersson. Total dietary fiber determined as neutral sugar residues, uronic acid residues, and Klason lignin (the Uppsala method): Collaborative study. *J AOAC Int*, 78(4):1030–1044, 1995.

[62] H. Boskov Hansen, M. Andreasen, M. Nielsen, L. Larsen, B.K Knudsen, A.S. Meyer, L.P. Christensen, and Å. Hansen. Changes in dietary fibre, phenolic acids and activity of endogenous enzymes during rye bread-making. *Eur. Food Res. Technol.*, 214(1):33–42, 2002.

[63] R. Andersson, G. Fransson, M. Tietjen, and P. Åman. Content and molecular-weight distribution of dietary fiber components in whole-grain rye flour and bread. *J. Agric. Food Chem.*, 57(5):2004–2008, 2009.

[64] T. Kujala. Rye: Nutrition, health and functionality. rakennuspaino oy, helsinki, finland. `http://rye.vtt.fi/`, 2000.

[65] H. Boskov Hansen, C.V. Rasmussen, K.E. Knudsen, and Å. Hansen. Effect of genotype and harvest year on content and composition of dietary fibre in rye (secale cereale l.) grain. *J. Sci. Food Agric.*, 83:76–85, 2003.

[66] K.H. Liukkonen, R.L. Heiniö, M. Salmenkalloo-Marttila, K. Autio, K. Katina, and K. Poutanen. Rye. In Y. H. Hui, editor, *Bakery Products: Science and Technology*, pages 109–122. Blackwell Publishing, 2006.

[67] S. Karppinen, O. Myllymäki, P. Forssell, and K. Poutanen. Fructan content of rye and rye products. *Cereal Chem*, 80(2):168–171, 2003.

[68] A.M. Aura, O. Myllymäki, M. Bailey, and J.L. Penalvo. Interrelationships between carbohydrate type, phenolic acid and initial pH on in vitro conversion of enterolactone from rye lignans. In Hannu Salovaara, Fred Gates, and Maija Tenkanen, editors, *Dietary Fibre Components And Functions*, pages 235–245. Wageningen Academic Pub, 2007.

[69] M. Cyran and L. Saulnier. Macromolecular structure of water-extractable arabinoxylans in endosperm and wholemeal rye breads as factor controlling their extract viscosities. *Food Chem*, 131(2):667–676, 2012.

[70] K. Venema, A.A. de Graaf, and N.E.P. Deutz. Metabolic conversations of dietary carbohydrates by gut microbes. In Hannu Salovaara, Fred Gates, and Maija Tenkanen, editors, *Dietary Fibre Components And Functions*, pages 145–156. Wageningen Academic Pub, 2007.

[71] R. Shibata, M. Kimura, H. Takahashi, K. Mikami, Y. Aiba, H. Takeda, and Y. Koga. Clinical effects of kestose, a prebiotic oligosaccharide, on the treatment of atopic dermatitis in infants. *Clin. Exp. Allergy*, 39(9):1397–1403, 2009.

[72] M. Elleuch, D. Bedigian, O. Roiseux, S. Besbes, C. Blecker, and H. Attia. Dietary fibre and fibre-rich by-products of food processing: Characterisation, technological functionality and commercial applications: A review. *Food Chem*, 124(2):411–421, 2011.

[73] A. Caprez, E. Arrigoni, R. Amadò, and H. Neukom. Influence of different types of thermal treatment on the chemical composition and physical properties of wheat bran. *J. Cereal Sci.*, 4(3):233–239, 1986.

[74] C.V. Rasmussen, H. Boskov Hansen, Å. Hansen, and L.M. Larsen. ph-, temperature- and time-dependent activities of endogenous endo-β-d-xylanase, β-d-xylosidase and α-l-arabinofuranosidase in extracts from ungerminated rye (secale cereale l.) grain. *J. Cereal Sci.*, 34(1):49–60, 2001.

[75] L. Axelsson. Lactic acid bacteria. In S. Salminen, A. von Wright, and A. Ouwehand, editors, *Lactic Acid Bacteria*. Markel Dekker, Inc, 2007.

[76] J. Hauf, F.K. Zimmermann, and S. Müller. Simultaneous genomic overexpression of seven glycolytic enzymes in the yeast *Saccharomyces cerevisiae*. *Enzyme Microb. Technol.*, 26(9-10):688–698, 2000.

[77] M. Cyran, C. Courtin, and J. Delcour. Structural features of arabinoxylans extracted with water at different temperatures from two rye flours of diverse breadmaking quality. *J. Agric. Food Chem.*, 51(15):4404–4416, 2003.

[78] E. Makarska, A. Ciołek, and W. Kociuba. Composition of gluten proteins and quality parameters of winter triticale hybrids. *Polish J. Food Nutrition Sci.*, 58(3):341–344, 2008.

[79] K. Buksa, A. Nowotna, W. Praznik, H. Gambuś, R. Ziobro, and J. Krawontka. The role of pentosans and starch in baking of wholemeal rye bread. *Food Res. Int.*, 43(8):2045–2051, 2010.

[80] C. M. Courtin and J. A. Delcour. Arabinoxylans and endoxylanases in wheat flour bread-making. *J. Cereal Sci.*, 35(3):225–243, 2002.

[81] M. Jankiewicz and J. Michniewicz. The effect of soluble pentosans isolated from rye grain on staling of bread. *Food Chem*, 25(4):241–249, 1987.

[82] M. Izydorczyk and C. Biliaderis. Cereal arabinoxylans: Advances in structure and physicochemical properties. *Carbohydr Polym*, 28(1):33–48, 1995.

[83] Z. Czuchajowska. Differential scanning calorimetry, water activity, and moisture contents in crumb center and near-crust zones of bread during storage. *Cereal Chem*, 66(4):NA, 1989.

[84] A. H. Sourki, M. G. Davoodi, F. T. Yazdi, S. A. Mortazavi, M. Karimi, R. Jahromi, and A. Pourfarzad. Staling and quality of iranian flat bread stored at modified atmosphere in different packaging. *World Acad. Sci. Eng. Technology*, 70:NA, 2012.

[85] A. Schiraldi and D. Fessas. Mechanism of staling. In P. Chinachoti and Y. Vodovotz, editors, *Bread Staling*. CRC Press, 2001.

[86] S. Hizukuri, Y. Takeda, M. Yasuda, and A. Suzuki. Multi-branched nature of amylose and the action of debranching enzymes. *Carbohydr. Res.*, 94(2):205–213, 1981.

[87] C.C. Maningat. Wheat starch: Production, properties , modification and uses. In James N. BeMiller and Roy L. Whistler, editors, *Starch: Chemistry and Technology*, pages 441–510. Academic Press, 3rd edition, March 2009.

[88] J.L. Jane. Structural features of starch granules II. In James N. BeMiller and Roy L. Whistler, editors, *Starch: Chemistry and Technology*, pages 193–236. Academic Press, 3rd edition, March 2009.

[89] K. J. Zeleznak and R. C. Hoseney. Characterization of starch from bread aged at different temperatures. *Starch - Starke*, 39(7):231–233, 1987.

[90] K. Autio and A.C. Eliasson. Rye starch. In James N. BeMiller and Roy L. Whistler, editors, *Starch: Chemistry and Technology*, pages 579–587. Academic Press, 3rd edition, March 2009.

[91] J. Blazek and E. Gilbert. Application of small-angle X-ray and neutron scattering techniques to the characterisation of starch structure: A review. *Carbohydr Polym*, 85(2):281–293, 2011.

[92] R. Tester, J. Karkalas, and X. Qi. Starch—composition, fine structure and architecture. *J. Cereal Sci.*, 39(2):151–165, 2004.

[93] C. P. Berry. The characterization of triticale starch and its comparison with starches of rye, durum, and HRS wheat. *Cereal Chem*, 48(4):NA, 1971.

[94] S. Gomand, T. Verwimp, H. Goesaert, and J. Delcour. Structural and physicochemical characterisation of rye starch. *Carbohydr. Res.*, 346(17):2727–2735, 2011.

[95] I. Farhat. Applications of NMR in the studies of starch systems. In G. Webb, editor, *Modern Magnetic Resonance*. Springer, 2008.

[96] A. Imberty, A. Buléon, V. Tran, and S. Péerez. Recent advances in knowledge of starch structure. *Starch/Starke*, 43(10):375–384, 1991.

[97] E. Svensson and A. Eliasson. Crystalline changes in native wheat and potato starches at intermediate water levels during gelatinization. *Carbohydr Polym*, 26(3):171–176, 1995.

[98] S. Cauvain. *Bread Making: Improving Quality*. Woodhead Publishing, 2003.

[99] S. Perez, P.M. Baldwin, and D.J. Gallant. Structural features of starch granules I. In James N. BeMiller and Roy L. Whistler, editors, *Starch: Chemistry and Technology*, pages 149–192. Academic Press, 3rd edition, March 2009.

[100] A. Imberty, H. Chanzy, S. Pérez, A. Buléon, and V. Tran. The double-helical nature of the crystalline part of a-starch. *J. Mol. Biol.*, 201(2):365–378, 1988.

[101] D. Popov, M. Burghammer, A. Buléon, N. Montesanti, J. L. Putaux, and C. Riekel. α-amylose single crystals: Unit cell refinement from synchrotron radiation microdiffraction data. *Macromolecules*, 39(10):3704–3706, 2006.

[102] H. Tang and B. Hills. Use of ^{13}C MAS NMR to study domain structure and dynamics of polysaccharides in the native starch granules. *Biomacromolecules*, 4(5):1269–1276, 2003.

[103] T. Wang, T. Bogracheva, and C. Hedley. Starch: As simple as a, b, c? *J. Exp. Bot.*, 49(320):481–502, 1998.

[104] H. Belitz, W. Grosch, and P. Schieberle. Structure and properties of starch granules. In H. Belitz, W. Grosch, and P. Schieberle, editors, *Food Chemistry*. Springer-Verlag, 4th edition, 2009.

[105] W. Ratnayake and D. Jackson. Gelatinization and solubility of corn starch during heating in excess water: New insights. *J. Agric. Food Chem.*, 54(10):3712–3716, 2006.

[106] C. Primo-Martín, N. H. van Nieuwenhuijzen, R. J. Hamer, and T. van Vliet. Crystallinity changes in wheat starch during the bread-making process: Starch crystallinity in the bread crust. *J. Cereal Sci.*, 45(2):219–226, 2007.

[107] A. Guarda, C. M. Rosell, C. Benedito, and M. J. Galotto. Different hydrocolloids as bread improvers and antistaling agents. *Food Hydrocolloids*, 18(2):241–247, 2004.

[108] S. Hug-Iten, F. Escher, and B. Conde-Petit. Structural properties of starch in bread and bread model systems: Influence of an antistaling α-amylase. *Cereal Chem*, 78(4):421–428, 2001.

[109] K. Katina, M. Salmenkallio-Marttila, R. Partanen, P. Forssell, and K. Autio. Effects of sourdough and enzymes on staling of high-fibre wheat bread. *Lebensm. Wiss. Technol.*, 39(5):479–491, 2006.

[110] M. Baik, L. C. Dickinson, and P. Chinachoti. Solid-state ^{13}C CP/MAS NMR studies on aging of starch in white bread. *J. Agric. Food Chem.*, 51(5):1242–1248, 2003.

[111] M. Siljestrom. Effects on polysaccharides during baking and storage of bread–in vitro and in vivo studies. *J. Dairy Res.*, 65(1):1–8, 1988.

[112] T. Verwimp, G. E. Vandeputte, K. Marrant, and J. A. Delcour. Isolation and characterisation of rye starch. *J. Cereal Sci.*, 39(1):85–90, 2004.

[113] E. Kirchhoff and P. Schieberle. Quantitation of odor-active compounds in rye flour and rye sourdough using stable isotope dilution assays. *J. Agric. Food Chem.*, 50(19):5378–5385, 2002.

[114] A. Hansen and P. Schieberle. Generation of aroma compounds during sourdough fermentation: Applied and fundamental aspects. *Trends Food Sci. Technol.*, 16(1-3):85–94, 2005.

[115] Y. Zhang, X. Li, C. Lo, and S. Guo. Characterization of the volatile substances and aroma components from traditional soypaste. *Molecules*, 15(5):3421–3427, 2010.

[116] Y. Wang and C. Ho. Comparison of 2-acetylfuran formation between ribose and glucose in the maillard reaction. *J. Agric. Food Chem.*, 56(24):11997–12001, 2008.

[117] R. Venskutonis, R. Vasiliauskait, A. Galdikas, and A. Šetkus. Use of gc-headspace and "electronic nose" for the detection of volatile compounds from glucose–glycine maillard reaction. *Food Control*, 13(1):13–21, 2002.

[118] G. López-Carballo, D. Cava, J. Lagarón, R. Catalá, and R. Gavara. Characterization of the interaction between two food aroma components, alpha-pinene and ethyl butyrate, and ethylene-vinyl alcohol copolymer (evoh) packaging films as a function of environmental humidity. *J. Agric. Food Chem.*, 53(18):7212–7216, 2005.

[119] T. Maeda, J. Kim, Y. Ubukata, and N. Morita. Analysis of volatile compounds in polished-graded wheat flour bread using headspace sorptive extraction. *Eur. Food Res. Technol.*, 228(3):457–465, 2009.

[120] C. Johansson, M. Siljeström, and N. Asp. Dietary fibre in bread and corresponding flours–formation of resistant starch during baking. *Z Lebensm Unters Forsch*, 179(1):24–28, 1984.

[121] KEGG. Kyoto encyclopedia of genes and genomes, release 58, retrieved april 15th, 2012. `http://www.genome.jp/kegg/pathway.html`, 2012.

[122] S. Hug-Iten, S. Handschin, B. Conde-Petit, and F. Escher. Changes in starch microstructure on baking and staling of wheat bread. *Lebensm. Wiss. Technol.*, 32(5):255–260, 1999.

[123] J. Silverio, E. Svensson, A. Eliasson, and G. Olofsson. Isothermal microcalorimetric studies on starch retrogradation. *J Therm Anal Calorim*, 47(5):1179–1200, 1996.

[124] J. Zhang, J. Zhang, L. Lin, T. Chen, J. Zhang, S. Liu, Z. Li, and P. Ouyang. Dissolution of microcrystalline cellulose in phosphoric acid–molecular changes and kinetics. *Molecules*, 14(12):5027–5041, 2009.

[125] R. J. Henry. Pentosan and (1→3),(1→4)-β-glucan concentrations in endosperm and wholegrain of wheat, barley, oats and rye. *J. Cereal Sci.*, 6(3):253–258, 1987.

[126] J. P. Roubroeks, R. Andersson, and P. Åman. Structural features of (1→3),(1→4)-β-D-glucan and arabinoxylan fractions isolated from rye bran. *Carbohydr Polym*, 42(1):3–11, 2000.

CURRICULUM VITAE

CURRICULUM VITAE

Name	Anna Mihhalevski	
Date and place of birth	04.05.1076, Tallinn, Estonia	
Institution	Competence Center of Food and Fermentation Technologies	
E-mail, address	anna@tftak.eu, AS TFTAK, Akadeemia tee 15, Tallinn 12618, Estonia	
Citizenship	Estonian	
Education	2003 – Present	PhD studies in Tallinn University of Technology, Bio- and Food Technology
	1999 – 2002	MSc studies in Tallinn University of Technology, Bio- and Food Technology
	1993 – 1998	Engineer studies in Tallinn University of Technology, Department of Food Processing
	1983 –1993	Tallinn Secondary School no. 53
Professional experience	09/2005 – Present	Competence Center of Food and Fermentation Technologies, project manager
	04/2002 – 08/2005	Agriculture Research Centre, Laboratory for Residue and Contaminants, Chief Specialist
Academy degree year	Master of Natural Sciences	
Year and site of earning the degree	Tallinn University of Technology, 2002	
Current grant funding	Project EU29994 – support for Competence Center of Food and Fermentation Technologies ETF9189 – Analysis of B-complex vitamin metabolom in food production chain ETF9417 – Biodiversity and stability of lactic acid bacteria and yeast during continues propagation of rye sourdough SF0140090s08 – Food systems biology and physics	

ELULOOKIRJELDUS

Ees- ja perekonnanimi	Anna Mihhalevski	
Sünniaeg ja -koht	04.05.1076, Tallinn, Eesti	
Töökoht	Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus	
E-mail, aadress	anna@tftak.eu, AS TFTAK, Akadeemia tee 15, Tallinn 12618, Eesti	
Kodakondsus	Eesti	
Hariduskäik	2003 – …	Doktoriõpe Tallinna Tehnikaülikoolis, Bio- ja toiduainetetehnoloogia õppesuunas
	1999 – 2002	Magistriõpe Tallinna Tehnikaülikoolis, Bio- ja toiduainetetehnoloogia õppesuunas
	1993 – 1998	Tallinna Tehnikaülikool, Toiduainete töötlemise õppesuund
	1983 –1993	Tallinna 53. Keskkool
Teenistuskäik	09/2005 – …	Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus, projektijuht
	04/2002 – 08/2005	Põllumajandusuuringute Keskus, Jääkide ja saasteainete laboratoorium, peaspetsialist
Teaduskraad	Loodusteaduste magister	
Teaduskraadi välja andnud asutus, aasta	Tallinna Tehnikaülikool, 2002	
Jooksvad grandid	Projekt EU29994 – finantstoetus Toidu- ja Fermentatsioonitehnoloogia Arenduskeskusele ETF9189 – B-komplex vitamiinide metaboloomi analüüs toidu tootmise ahelas ETF9417 – Piimhappebakterite ja pärmide mitmekesisus ja stabiilsus rukkitaigna uuendamisel SF0140090s08 – Toidu süsteembioloogia ja füüsika	

APPENDICES

PUBLICATION I

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ORIGINAL ARTICLE

Growth characterization of individual rye sourdough bacteria by isothermal microcalorimetry

A. Mihhalevski[1,2], I. Sarand[1,2], E. Viiard[1,2], A. Salumets[1,2] and T. Paalme[1,2]

1 Competence Center of Food and Fermentation Technologies, Tallinn, Estonia
2 Department of Food Processing, Tallinn University of Technology, Tallinn, Estonia

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Correspondence
Anna Mihhalevski, Competence Center of Food and Fermentation Technologies, Akadeemis tee 15b, Tallinn, 12618, Estonia.
E-mail: anna@tftak.org

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Abstract

Aims: The present work tests the feasibility of the isothermal microcalorimetry method to study the performance of individual lactic acid bacteria during solid-state fermentation in rye sourdough. Another aim was to elucidate the key factors leading to the formation of different microbial consortia in laboratory and industrial sourdough during continuous backslopping propagation.
Methods and Results: Strains of the individual LAB isolated from industrial and laboratory sourdough cycle were grown in 10 kGy irradiated rye dough in vials of an isothermal calorimeter and the power–time curves were obtained. Sugars, organic acids and free amino acids in the sourdough were measured. The OD–time curves of the LAB strains during growth in flour extract or MRS (De Man, Rogosa and Sharpe) broth were also determined. The maximum specific growth rates of *Lactobacillus sakei*, *Lactobacillus brevis*, *Lactobacillus curvatus and Leuconostoc citreum* strains that dominated in backslopped laboratory sourdough were higher than those of *Lactobacillus helveticus*, *Lactobacillus panis*, *Lactobacillus vaginalis*, *Lactobacillus casei* and *Lactobacillus pontis* strains originating from industrial sourdough. Industrial strains had higher specific growth rates below pH 4·8. It was supposed that during long-run industrial backslopping processes, the oxygen sensitive species start to dominate because of the O_2 protective effect of rye sourdough.
Conclusions: Measurements of the power–time curves revealed that the LAB strains dominating in the industrial sourdough cycle had better acid tolerance but lower maximum growth rate and oxygen tolerance than species isolated from a laboratory sourdough cycle.
Significance and Impact of the Study: Isothermal microcalorimetry combined with chemical analysis is a powerful method for characterization of sourdough fermentation process and determination of growth characteristics of individual bacteria in sourdough.

Introduction

The production of sourdough bread can be traced back to ancient times. Sourdough is a mixture of flour and water that is fermented with lactic acid bacteria (De Vuyst and Neysens 2005; Rosenquist and Hansen 2000). Sourdough fermentation improves volume, texture, flavour and nutritional value of the bread. It also retards the staling process of bread and protects it from mould and bacterial spoilage (Katina *et al.* 2002; De Vuyst and Vancanneyt 2007; Hammes *et al.* 2005). Hundreds different types of traditional sourdough breads exist in Europe. They differ in the type of flour, additives, applied technology and sourdough microbiota (De Vuyst and Vancanneyt 2007). The latter consists from the specifically adapted lactic acid bacteria (LAB) and yeasts (Corsetti and Setanni 2007; De Vuyst *et al.* 2009).

Stable and reproducible composition and activity of the sourdough microflora is indispensable to achieve a constant quality of sourdough bread (De Vuyst and

Neysens 2005). Consecutive microbial re-inoculation, or so called 'backslopping', of the micro-organisms from a previous sourdough batch is used to maintain the adapted microbial flora, which is selected in the process applied (Häggman and Salovaara 2008). The consortia of bacteria that develop in the sourdough during repeated solid-state fermentations originate from the starter culture, flour, bakery environment or humans. Establishment of a stable sourdough ecosystem is a consecutive process correlating with acid stress and carbohydrate and amino acid (AA) metabolism (Van der Meulen *et al.* 2007).

Evaluation of the growth properties of individual bacterial strains in the sourdough is a complicated task as no precise method exists for microbial biomass quantification in the solid state. The plating method is laborious, requires synthetic media selection (Vera *et al.* 2009) and might not be suitable for some fastidious lactic acid bacteria species present in industrial sourdough (De Vuyst and Vancanneyt 2007). In the case of a food matrix like rye dough, the application of the indirect methods measuring bacterial DNA, RNA-s, proteins (Abd-Aziz *et al.* 2008) or the concentration of cell wall components is quite complicated. Alternatively, measurements of metabolites and CO_2 formation are often used for the estimation of microbial growth during solid-state fermentations (Terebiznik and Pilosof 1999). Although the growth yields may depend significantly on growth conditions, parameters like O_2 consumption, CO_2, acid and heat production provide a valuable tool for continuous on-line measurement of the microbial growth in the solid state.

Microcalorimetry has been used for the experimental study of bacterial growth during the last 50 years (Wadsö 2002; Braissant *et al.* 2010). The first experiments were carried out with *Streptococcus faecalis* and it was shown that the rate of heat production was coupled with the rate of biomass increase (Forrest and Walker 1963). The measurement of different growth parameters together with the heat production rate has made it clear that the shape of the power–time curve is influenced by the type of metabolic activity and can be related to the different physiological states of bacteria (Gustafsson 1991; Wadsö 1997; Menert *et al.* 2001). Recently, isothermal microcalorimetry (IMC) has been used as a universal tool for real-time evaluation of processes rates during cell culturing (Fang and Hedin 2006; Kabanova *et al.* 2009) including cell replication rates and effects of biocides on microbial activity (Vandenhove 1998; Yi *et al.* 2000; Fang and Hedin 2006; De San *et al.* 2007; Von Ah *et al.* 2008; Wadsö and Galindo 2009). IMC experiments are entirely passive. The specimen is not disturbed during measurement and can be removed from the calorimeter and evaluated by other means (Wadsö 2002; Von Ah *et al.* 2008; Braissant *et al.* 2010) such as determining the plate counts, acid and ethanol concentrations, pH, etc. (Kabanova *et al.* 2009). All this makes high throughput IMC applications useful in the analysis of microbial processes in food, particularly during anaerobic processes like cheese ripening and sourdough fermentation.

The technological parameters of rye sourdough bread production differ significantly in different bakeries thus causing variations in microbial consortia of sourdoughs (Rosenquist and Hansen 2000; De Vuyst *et al.* 2009). To understand the factors (mechanisms) that determine the dominance of different bacterial species in different continuously backslopped sourdoughs, the bacteria should be studied in their natural environment of sourdough as the growth characteristics determined in artificial liquid media and even in flour extract might be misleading. For example, it has been shown that starch and gluten in hydrated flour are able to reversibly adsorb oxygen (Xu 2001; Joye *et al.* 2009) thus leading to low levels of dissolved oxygen in dough.

Except for soils science (Wadsö *et al.* 2004; Oriol *et al.* 1987), very few attempts have been made to grow cells on solid (or 'semi-solid') substrate in a microcalorimetric ampoule.

The aim of the present work was to demonstrate the feasibility of the IMC method for the study of performance of individual lactic acid bacteria (LAB) during solid-state fermentation in rye dough and to elucidate the key factors leading to formation of different microbial consortia in the laboratory and industrial sourdough during continuous backslopping propagation.

Materials and methods

Bacterial strains used

The bacterial strains used in this work are listed in Table 1. Strains belonging to the *Lactobacillus helveticus*, *Lactobacillus panis*, *Lactobacillus vaginalis*, *Lactobacillus casei* and *Lactobacillus pontis* species were isolated from the industrial rye sourdough cycle, while strains belonging to the *Lactobacillus sakei*, *Lactobacillus brevis*, *Lactobacillus curvatus and Leuconostoc citreum* species were isolated from the spontaneously started laboratory rye sourdough (Viiard *et al.*, in preparation). A local bakery supplied the industrial sourdough sample. It was taken from a 6-month-old sourdough cycle with a backslopping renewal of 1 : 10 every 10 h at 30°C. Laboratory sourdough was fermented at 20°C with a daily backslopping of 1 : 10 during 18 days. The strains were maintained in stock as 25% glycerol cultures at −80°C and re-cultivated

Table 1 Bacteria strains used in this work

Bacteria strains	Source of isolation
Homofermentative LAB	
Lactobacillus casei N726	Industrial rye sourdough
Lactobacillus curvatus 0E12-11	Spontaneously started laboratory sourdough
Lactobacillus helveticus N92	Industrial rye sourdough
Lact. helveticus E96	Industrial rye sourdough
Lactobacillus sakei 0E12-10	Spontaneously started laboratory sourdough
Heterofermentative LAB	
Lactobacillus brevis 0E12-37	Spontaneously started laboratory sourdough
Leuconostoc citreum 3N18-10	Spontaneously started laboratory sourdough
Lactobacillus panis N915	Industrial rye sourdough
Lactobacillus pontis N131	Industrial rye sourdough
Lactobacillus vaginalis N1113	Industrial rye sourdough
Lact. vaginalis E1113	Industrial rye sourdough

anaerobically in MRS broth (Lab M, Bury, UK) 48 h at 30°C for the dough fermentation experiments.

Flours

The rye flour (type R1370) used for both the spontaneous fermentation in the laboratory and in the sourdough process in industry was derived from the same source, Tartu Grain Mill Ltd (Tartu, Estonia). Part of the flour was irradiated in sealed 400-g-plastic Stomacher bags at doses 1, 4, 10 or 20 kGy using dosimetric system GEX WinDose (Centennial, CO, USA). The flour extract used in ''Bioscreen'' experiments was prepared from 10 kGy irradiated rye flour by mixing with water in the ratio 1 : 2, incubating overnight at +4°C and clarifying the extract by centrifugation.

Sourdough fermentation

The 10 kGy irradiated rye flour (150 g) and bacterial suspension prepared from individual LAB strains (5×10^6–5×10^7 CFU ml^{-1}) in 0·5% of NaCl were mixed to a 1 : 1 ratio in Stomacher 400 Circulator (Seward Ltd, Worthing, UK) bags for 15 min at 100 rev min^{-1}. At least three aliquots (*c.* 2·5 g) of the dough were transferred into 3-ml microcalorimetric vials and incubated in the Thermal Activity Monitor TAM III (TA Instruments, New Castle, DE, USA) at 30°C. To test the effect of oxygen on heat flow, the oxygen concentration was decreased by removing air from vial headspace. The remaining dough (about 400 g) was incubated in the stomacher bags in the Environmental Test Camber (Sanyo Electric Biomedical Co. Ltd, Morigushi, Japan) at 30°C in parallel to isothermal calorimetry analyses.

Analytical methods

Plate counts

For enumeration of bacteria, five grams of sourdough was suspended in 45 ml of 0·5% NaCl solution and serial dilutions were plated on MRS agar plates. Bacteria were counted after 48 h incubation at 30°C in anaerobic and aerobic growth conditions.

The power–time curves of dough samples were resampled with a 5-min interval starting from 1 h after flour hydratation using Thermal Activity Monitor TAM III (TA Instruments).

The OD–time curves of the LAB strains during growth in flour extract or MRS broth were determined using Bioscreen C (Oy Growth Curves Ab Ltd, Helsinki, Finland) at 30°C. At least three parallel measurements were performed with 15-min intervals. To obtain anaerobic conditions, the micro-plates with bacteria suspensions were incubated for 15 min in a nitrogen atmosphere and sealed in it prior to measurement.

Metabolite analysis

The concentration of sugars and organic acids in sourdough was measured from three parallel dough samples after extraction of samples with 20 volumes of water – isopropanol mixture (1 : 1) and diluting the extract in ratio 1 : 10 with 0·008 N sulfuric acid (Rathburn Chemicals Ltd, Walkerburn, UK). The latter was also used as the eluent in HPLC (Waters, USA) analysis. The analysis was performed at a flow rate 0·6 ml min^{-1} at 35°C using an Aminex HPX 87H column (BioRad, Philadelphia, PA, USA). A Refractive Index Detector (Waters 2414, Milford, MA, USA) was used for detection of disaccharides (sucrose + maltose), glucose, fructose and mannitol, lactic acid and acetic acid.

Free AAs were analysed from three parallel freeze-dried and powdered sourdough samples. The samples (about 0·5 g) were homogenized with 5 ml water (Milli Q, Millipore SAS, Molsheim, France), centrifuged and supernatant was diluted with isopropanol (1 : 1). Ten microlitres of homogenized and centrifuged samples were filtered and derivatized with 70 μl of AccQ.Taq Ultra borate buffer and 20 μl AccQ.Taq reagent (Waters), heated at 55°C in a ThermoBlock TDB-120 (Biosan, Riga, Latvia) and analysed by UPLC (Acquity™, Waters) equipped with a PDA detector at 260 nm.

pH and acidity of sourdough were determined using DL22 titrator (Mettler Toledo GmbH, Schwerzenbach, Switzerland). For acidity measurement, 5 g of sourdough sample was homogenized with 50 ml water using PT 2100 homogenizer (Kinematica AG, Luzern, Switzerland). Total acidity was calculated as mmol NaOH required for titration of 1 g of dry mass of dough up to pH = 8·5.

Dry weight of flour and dough was measured by Halogen Moisture Analyser HR83 (Mettler Toledo). All data presented are given per dry matter (DM).

Calculation of growth parameters

The specific growth rate of bacteria μ is equal to:

$$\mu = \frac{dX}{dt \times X} \quad (1)$$

where X is the amount of biomass. The biomass can be measured directly as dry weight or indirectly:

$$X = k \times OD; \qquad X = Y_{XZ} \times Z; \qquad X = \frac{Y_{XZ} \times F}{\mu} \quad (2)$$

where k is biomass calibration constant, Y_{XZ} is the biomass yield based on production of Z (CO_2, heat, acid, etc.) and F is the rate of Z production ($F = dZ/dt$).

The specific growth rate μ can be calculated from OD–time plot if k is constant as:

$$\mu = \frac{d(OD)}{dt \times OD} \quad (3)$$

If the biomass yield Y_{XZ} is constant then specific growth rate can be also calculated as:

$$\mu = \frac{d(Z)}{dt \times Z} = \frac{F}{Z} \quad (4)$$

and if the both biomass yield Y_{XZ} and μ are constant then the specific growth rate can be calculated as:

$$\mu = \frac{d(F)}{dt \times F} \quad (5)$$

where $dZ/dt = F$ is production (flow) rate. Solving eqns (1) and (3–5) we will derive:

$$\begin{aligned} X_t &= X_0 \times e^{\mu t} \qquad OD_t = OD_0 \times e^{\mu t} \\ Z_t &= Z_0 \times e^{\mu t} \qquad F_t = F_0 \times e^{\mu t} \end{aligned} \quad (6)$$

The specific growth rate can be calculated from calorimetric heat flow data as:

$$\mu = \frac{\ln(F_t) - \ln(F_0)}{t} \quad (7)$$

were F_0 is the heat flow in starting and F_t in final exponential growth point and t is the time between those points.

In the current study, to determine the range of exponential growth phase, the value μ_t was calculated from power–times at 5-min intervals:

$$\mu_t = \frac{\ln(F_t - B_t) - \ln(F_{t-1} - B_{t-1})}{\Delta t} \quad (8)$$

where P_t is the total heat production rate of the sample measured calorimetrically and B_t is the heat flow of background processes occurring in rye dough independent from microbial growth.

If the μ_t values remained constant ($ST_{DEV} < \pm 5\%$) at least during one generation time, then the μ_t value was considered to be equal to specific maximum growth rate μ_{exp}.

Supposing that biomass yield based on heat accumulation Y_{XH} remains constant, μ_t can be calculated also according to formula (4) where Z is equal to accumulated heat Q_t which can be calculated as

$$Q_t = Q_\tau + \int_\tau^t F t \, dt \quad (9)$$

where Q_τ is the apparent accumulated heat for 'first' exponential point. If the μ_{exp} is known, then the Q_τ value can be derived from formula (4) and:

$$\mu_t = \frac{F_t}{Q_t} \quad (10)$$

Equation (10) was used to predict of the behaviour of the specific growth rate after the exponential growth phase. In these calculations, it is assumed that the decrease in growth yield was not involved in the decrease in specific growth rate.

The specific growth rates from 'Bioscreen' data were calculated as:

$$\mu_t = \frac{\ln(OD_{t+1} - BO_{t+1}) - \ln(OD_{t-1} - BO_{t-1})}{2\Delta t} \quad (11)$$

where BO is the OD background (OD of not inoculated sample) at time-point t.

The heat yield based on acid formation Y_{HA} was calculated as:

$$Y_{HA} = \frac{Q_t - Q_0}{lac_t + ace_t - lac_0 - ace_0} \quad (12)$$

where lac_t and lac_0 designate lactate concentrations (mmol per g flour) and ace_0 and ace_t acetate concentrations. The zero subscript indicates the start of fermentation, while t indicates the end.

Concentrations of total acids A_t were calculated from formula (12) using the average Y_{HA} values for hetero- and homofermentative bacteria. With the corresponding values of A_t, pH_t values were obtained from titration curve of rye flour using lactic acid.

The theoretical values of heat production of stoichiometric equations were calculated according to standard transformed Gibbs energies of formation for biochemical reactants (Goldberg and Alberty 2010).

Results

The heat production after hydratation of rye flours

The power–time curves of freshly mixed dough samples prepared from nonirradiated and irradiated flour were measured during incubation at 30°C in the microcalorimeter (Fig. 1). During the first 5 h of incubation, the specific heat flow curves of the dough samples coincided. Later, the increase in heat flow of nonirradiated and 1 kGy irradiated flour was observed, while in case of 4, 10 and 20 kGy irradiated flours, the heat flow continued to decrease (Fig. 1).

The heat flow behaviour of 0 and 1 kGy irradiated samples can be explained by microbial growth. The initial plate count of bacteria in the dough samples prepared from nonirradiated flour was 10^5 CFU g^{-1} DM, while in 1 kGy irradiated samples, it was <200 CFU g^{-1}. After 24-h incubation, the plate counts were $1{\cdot}8 \times 10^8$ and 4×10^5 CFU g^{-1} DM, respectively, which is close to the detection limit assuming 2 pW per cell (James 1987).

The heat flow maximum in 1 kGy dough was lower and detected 15 h later than in dough prepared from nonirradiated flour (Fig. 1).

In 4, 10 and 20 kGy irradiated samples, the microbes could not be detected even after week of incubation (not shown).

Thus, based on the calorimetric and microbial data, the irradiation of flour by 4 kGy was sufficient to prevent microbial growth in dough for at least a week. The increase in the irradiation dose up to 20 kGy had no statistically significant effect on the heat flow curve. The total heat produced during 24-h incubation by samples prepared from 4, 10 or 20 kGy irradiated flour was $4{\cdot}6 \pm 0{\cdot}4$ J g^{-1} DM. The 10 kGy irradiated flour was chosen to study the growth characteristics of different isolates of lactic acid bacteria in sourdough.

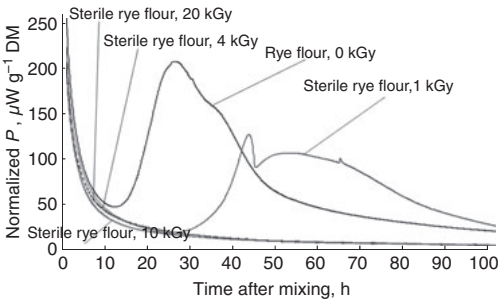

Figure 1 The power–time curves of rye dough prepared from nonirradiated (0 kGy), 1, 4, 10 and 20 kGy irradiated flour.

Metabolite analysis of the dough prepared from the 10 kGy irradiated flour revealed that sucrose, fructosanes and oligosaccharides as well as proteins were hydrolysed during incubation by indigenous enzymes of the rye flour (Table 2). After incubation for 24 h at 30°C, the fructose concentration increased by $38 \pm 1{\cdot}7$ μmol g^{-1} DM, glucose by 45 μmol g^{-1} DM, while the concentration of disaccharides (maltose + sucrose) decreased from 36 ± 1 to 28 ± 2 μmol g^{-1} DM. In addition, the concentration of total free AA increased from its initial value of 21·40–49·80 μmol g^{-1} DM (Table 2). According to standard transformed Gibbs energies of formation for biochemical reactants (Goldberg and Alberty 2010), the hydrolysis of 1,4-α-glucosidic linkages of maltose results in heat production close to $\Delta G^{o\prime}$ −20 and −29·5 J $mmol^{-1}$ in case of sucrose. This accounts for almost half of the produced heat ($20 \times 45 + 29{\cdot}5 \times 38 = 1{\cdot}9$ J g^{-1} DM). The remaining heat production might be related to breaking glycosidic bonds in carbohydrates and peptide linkages in proteins and peptides leading to the formation of about 28·4 μmol g^{-1} DM of AA (Table 2).

The heat production curves of the individual LAB in rye dough

The 10 kGy irradiated flour was suspended in 0·5% NaCl solutions containing strains of *Lact. helveticus*, *Lact. panis*, *Lact. vaginalis*, *Lact. casei*, *Lact. pontis*, *Lact. sakei*, *Lact. curvatus or Leuc. citreum* and was incubated in microcalorimetric vials at 30°C. The power–time curves of cultures were measured in the calorimeter, and the background of the dough matrix heat flow was subtracted (see Fig. 1). The resulting heat flow curves of different strains are shown on Fig. 2. By shape of the curve, the bacteria can be divided into two groups: with one (Fig. 2a,b) and two (Fig. 2c,d) heat flow maximums, which coincide with the division of LAB into homo- and heterofermentative species.

The strains of the homofermentative species, *Lact. sakei* 0E12-10 and *Lact. curvatus* 0E12-11, which were isolated from the laboratory 18 days backslopped sourdough had the earlier and higher heat flow maximums than those of *Lact. helveticus* N92, *Lact. helveticus* E96 and *Lact. casei* N726 strains isolated from the industrial sourdough which has been renewed by daily backslopping during 6 months.

Unlike homofermentative species, the representatives of heterofermentative LAB had two heat flow maximums (Fig. 2c,d). The height of the first maximum was about 1/3–1/2 from second maximum. The heat flow maximums were highest for *Leuc. citreum* 3N18-10 and *Lact. brevis* 0E12-37 strains derived from spontaneously started laboratory sourdough. However, both homo- and

Table 2 Formation of sugars, organic acids and free AAs during 24-h incubation at 30°C in the dough prepared from the 10 kGy irradiated flour with and without inoculation

	Amount in sourdough samples, μmol g^{-1} DM												
Compound	10 kGy rye flour, 0 h	10 kGy rye flour, 24 h	*Lactobacillus helveticus* N92	*Lact. helveticus* E96	*Lactobacillus casei* N 726	*Lactobacillus curvatus* 0E12-10	*Lactobacillus sakei* 0E12-10	*Lactobacillus brevis* 0E12-37	*Leuconostoc citreum* 3N18-10	*Lactobacillus panis* N915	*Lactobacillus pontis* N131	*Lactobacillus vaginalis* N1113	*Lact. vaginalis* E1113
Fructose	6	44	36	46	56	44	44	51	59	58	64	48	52
Glucose	6	51	53	64	67	63	61	49	57	43	58	50	56
Disaccharides	36	28	39	48	46	44	43	18	42	16	24	15	18
Lactate	0	0	98·9	158·9	130	101·1	110	83·3	67·8	138·9	70	84·4	75·6
Acetate	8·2	9	6·7	8·3	10	10	11·7	35	30	25	33·3	25	23·3
His	0·00	0·59	0·63	0·74	0·62	0·72	0·68	0·64	0·56	0·41	0·51	0·59	0·64
Asn	4·77	5·06	3·93	4·27	5·67	4·31	3·98	5·39	5·05	4·21	4·90	5·36	5·65
Ser	0·40	2·36	2·85	3·25	2·33	1·97	1·88	2·42	2·02	0·64	2·02	2·70	2·77
Gln	0·65	2·93	1·16	1·60	2·14	1·72	1·44	1·08	1·52	0·91	1·20	2·02	2·45
Arg	1·23	2·89	3·72	4·11	3·73	4·02	4·22	3·42	3·58	3·16	0·25	0·00	0·00
Gly	0·84	2·93	2·99	3·47	3·06	3·32	3·06	2·88	2·15	2·20	2·06	1·58	1·77
Asp	3·78	3·88	4·63	4·17	4·79	4·88	4·61	5·37	5·27	2·86	4·48	3·84	3·94
Glu	1·67	1·77	4·68	4·82	3·96	5·09	5·17	1·85	3·43	3·71	3·66	3·98	3·64
Thr	0·32	1·85	1·94	2·09	1·66	2·20	2·06	1·33	1·50	0·73	1·60	1·88	1·99
Ala	1·79	7·38	8·70	9·05	6·17	8·37	9·79	4·08	4·87	4·94	4·36	4·81	4·87
GABA	0·99	3·23	3·10	3·37	3·30	2·91	3·16	7·25	3·14	2·81	2·98	3·41	3·66
Pro	1·16	2·83	2·88	3·12	3·12	3·12	3·29	3·06	2·80	2·45	2·45	2·81	3·36
Orn	0·02	0·00	0·01	0·01	0·02	0·00	0·03	0·03	0·04	0·00	2·55	2·85	2·87
Cys-Cys	0·00	0·20	0·15	0·06	0·00	0·00	0·00	0·00	0·06	0·00	0·07	0·05	0·04
Lys	0·31	2·22	2·28	2·21	2·46	2·67	2·85	1·76	2·81	1·34	1·73	2·81	2·85
Tyr	0·34	0·28	0·48	0·60	0·77	1·09	1·10	1·07	0·85	0·46	0·61	0·81	0·72
Met	0·37	0·65	0·75	0·83	0·91	0·96	1·01	0·99	0·79	0·50	0·55	0·54	0·58
Val	0·72	2·77	3·16	3·53	3·04	3·82	3·73	3·46	2·89	2·61	2·61	3·20	3·10
Ile	0·45	1·44	1·57	1·84	1·44	2·01	1·85	1·85	1·27	1·20	1·23	1·57	1·55
Leu	0·77	2·70	4·16	4·76	4·50	5·34	5·58	5·42	3·79	3·72	3·30	4·25	3·86
Phe	0·49	1·30	1·20	1·54	2·17	2·48	2·62	2·63	1·89	1·73	1·53	1·83	1·67
Trp	0·33	0·51	0·57	0·64	0·69	0·72	0·77	0·75	0·65	0·56	0·55	0·66	0·64
TOTAL AA	21·39	49·79	55·54	60·09	56·54	61·70	62·83	56·74	50·95	41·14	45·20	51·54	52·63

AA, amino acid.

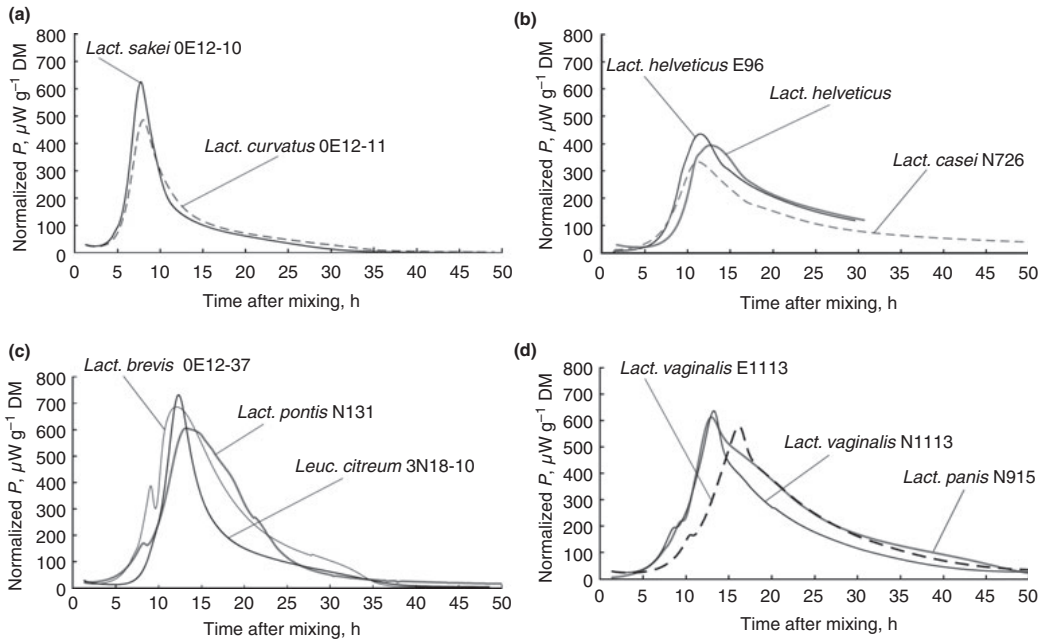

Figure 2 Power–time curves of sourdough samples inoculated with the different homofermentative (a,b) and heterofermentative (c,d) strains of LAB.

heterofermentative strains isolated from the industrial sourdough (*Lact. helveticus*, *Lact. panis*, *Lact. vaginalis*, *Lact. casei* and *Lact. pontis*) had higher heat flows at the later stages of rye dough fermentation.

In parallel to power–time curves, the pH, organic acids, AAs and concentration of sugars were measured in the sourdough samples at different time-points after mixing. The results revealed that inoculation of dough with lactic acid bacteria did not exhaust available carbon sources (maltose and glucose). In case of heterofermentative species, fructose was converted into mannitol. Also, all essential AAs were available in the dough at the end of fermentation (Table 2). Thus, the decrease in pH (see Table 3) was the most probable reason for the decrease in the heat flow and the reduction in growth after the maximum.

The final acidities of sourdoughs fermented by different individual bacteria remained in the range of 79–131 mmol acid per g DM and that of pH in range of 3·8–4·15 (Table 3). With the exception of *Lact. brevis* 0E12-37, the final acidities of sourdoughs prepared with the individual industrial sourdough bacteria (*Lact. helveticus*, *Lact. panis*, *Lact. vaginalis*, *Lact. casei* and *Lact. pontis*) were higher than the final acidities of sourdoughs prepared with *Lact. sakei*, *Lact. curvatus* and *Leuc. citreum* strains isolated from the spontaneously fermented rye flour. A statistically significant difference in the final acidity values between homo- and heterofermentative species was not observed. The increase in titratable acidity (mmol NaOH g flour^{-1}) correlated well with formation of organic acids lactate plus acetate. The ratio of titratable acidity to lactic + acetic acid formation in all sourdough samples was 0·95 ± 0·24 mol mol^{-1} after 24 h of incubation.

The accumulated heat yield Y_{HA} values based on acid formation (kJ mol acid^{-1}) were calculated by determining the concentrations of lactic and acetic acid in dough immediately after completing the fermentation with individual bacteria in calorimetric vials (Table 3). The average heat yield Y_{HA} was 89 ± 8 J mmol acid^{-1} for homo- and 167 ± 22 J mmol acid^{-1} for heterofermentative species. Those values were used for transforming the power–time curves into the specific growth rate acidity (pH) plots (Fig. 3).

The μ–pH plots shown on Fig. 3 revealed the significant differences in the growth behaviour of LAB species in sourdough. The *Lact. sakei*, *Lact. curvatus* and *Leuc. citreum* strains, which were derived from the laboratory spontaneous sourdough, had the higher specific growth rate at the onset with a near neutral pH compared

Table 3 Characterization of LAB strains used for the sourdoughs inoculation. Bacteria numbers in sourdough after 24-h fermentation, titratable acidity of sourdough after 24-h fermentation, pH of sourdough after 24-h fermentation, the heat yield per mol acid formed, the maximum specific growth rates of individual LABs measured by IMC in rye dough or by 'Bioscreen' in MRS and dough extract

Strain	24 h 10^9 CFU g^{-1} DM	24 h acidity, μmol g^{-1} DM	24 h pH	ΔHeat/(Δlac + Δace), J $mmol^{-1}$	μ_{max} dough, h^{-1}	μ_{max} MRS, h^{-1}	μ_{max} flour extract, h^{-1}
Homofermentative							
Lactobacillus helveticus N92*	0·41	103	3·95	99	0·62 ± 0·01	0·3	0·28
Lact. helveticus N92†				92	0·62 ± 0·01	0·65	0·65
Lact. helveticus N92‡		107	3·97	88	0·58 ± 0·01		
Lact. helveticus E96*	0·72	103	3·85	92	0·65 ± 0·03	0·35	0·31
Lact. helveticus E96†				78	0·66 ± 0·01	0·95	0·8
Lact. helveticus E96‡		123	3·86	77	0·58 ± 0·01		
Lactobacillus casei N726*	3·2	98	4·01	86	0·48 ± 0·01	0·43	0·45
Lact. casei N726†				86	0·48 ± 0·01	0·5	0·3
Lactobacillus curvatus 0E12-11*	2·1	87	3·94	100	0·84 ± 0·06	0·95	0·75
Lactobacillus sakei 0E12-10*	1·2	79	3·91	88	0·93 ± 0·01	0·83	0·68
Lact. sakei 0E12-10†						0·95	0·55
Average				89 ± 8			
Heterofermentative							
Lactobacillus panis N915*	8·5	112	3·91	140	0·46 ± 0·01	NG	0·31
Lact. panis N915†				176	0·46 ± 0·01	0·83	0·66
Lactobacillus vaginalis N1113*	2·7	122	4·01	133	0·53 ± 0·03	NG	0·2
Lact. vaginalis N1113†				151	0·53 ± 0·01	0·64	0·66
Lact. vaginalis E1113*	2·2	112	4·6	188	0·48 ± 0·01	NG	0·28
Lact. vaginalis E1113†				204	0·48 ± 0·01	0·3	0·4
Lactobacillus pontis N131*	0·68	114	4·05	172	0·45 ± 0·01	0·21	0·27
Lact. pontis N131†				168	0·44 ± 0·01		0·23
Lactobacillus brevis 0E12-37*	3·9	131	3·8	172	0·61 ± 0·02	0·68	0·78
Leuconostoc citreum 3N18-10*	2·5	94	4·15	181	0·83 ± 0·01	0·77	0·58
Leuc. citreum 3N18-10†						0·95	0·62
Average				167 ± 22			

DM, dry matter; IMC, isothermal microcalorimetry.
*Aerobic incubation conditions.
†Anaerobic incubation conditions.
‡24-h preincubated sterile dough, aerobic incubation conditions.

to 'industrial' strains. However, the strains of *Lact. vaginalis*, *Lact. panis*, *Lact. pontis*, *Lact. helveticus* isolated from the industrial sourdough clearly had higher specific growth rates at pH values less than 4·8 units compared to laboratory counterparts.

In parallel to the calorimetric determination of the maximum bacterial growth rates in sourdough, the maximum specific growth rates for the same strains were determined in MRS and sourdough extract using the 'Bioscreen' apparatus, which enabled a more direct (optical density) measurement of specific growth rate. The results showed that in conventional aerobic conditions of 'Bioscreen' experiments, the industrial sourdough strains *Lact. helveticus* N92, *Lact. helveticus* E96, *Lact. vaginalis* N1113 and E1113, *Lact. panis* N915 and *Lact. pontis* N131 strains had at least a twofold lower maximum specific growth rate in rye flour extract or MRS broth than measured calorimetrically in dough. In the case of *Lact. casei* N726, *Lact. brevis* 0E12-37, *Lact. curvatus* 0E12-11, *Lact. sakei* 0E12-10 and *Leuc. citreum* 3N18-10 isolated from laboratory sourdough, the specific growth rates measured in conventional aerobic conditions coincided with those of measured calorimetrically (Table 3). These results show that shaking and exposure to oxygen in liquid media negatively affected the growth of bacteria isolated from industrial sourdough but not those isolated from laboratory sourdough.

The negative effect of oxygen on the growth of industrial species in liquid media was confirmed in the experiments carried out in anaerobic conditions using 'Bioscreen'. All LAB tested showed maximum growth rate comparative with corresponding values which were determined calorimetrically (Table 3). Generally, both homo- and heterofermentative bacteria tested grew in anaerobic conditions with the same rate or faster in MRS broth than in flour extract.

In different from 'Bioscreen' experiments, the removal of the air from headspace of calorimetric vials did not

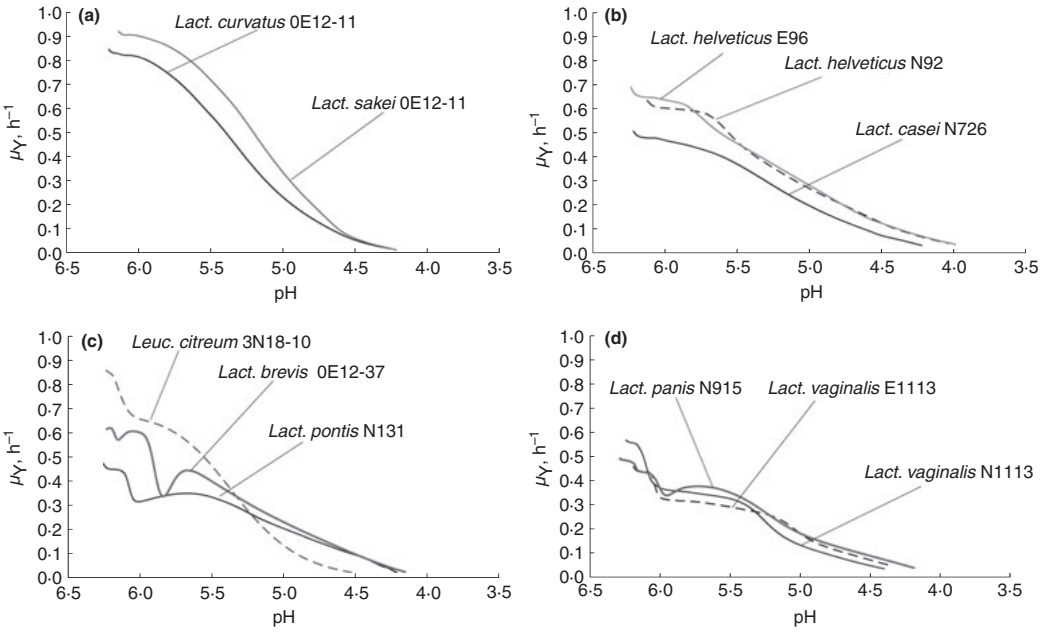

Figure 3 The dependence of LAB specific growth rate in sourdough from pH. (a,b) – homofermentative species, (c,d) heterofermentative species.

have a positive effect on the LAB-specific growth rate measured by IMC (Table 3). The absence of any effect might be explained by incomplete removal of oxygen from the system. The effect of oxygen on the growth of sourdough bacteria requires a separate study.

To decrease the background effect of the dough matrix on heat flow, the preincubation of rye dough during 24 h at 30°C before bacterial inoculation was performed in case of two *Lact. helveticus* strains. The dough preincubation reduced the background-related to enzymatic processes in flour more than 10 times (see Fig. 1) and thus significantly improved the preciseness of microbial heat flow calculation in the exponential growth phase. The maximum growth rates of *Lact. helveticus* N92 and E96 strains determined in the preincubated dough were 5-10% lower (μ_{max} = 0·58 ± 0·01 h^{-1} for the both strains) than corresponding values (0·62 and 0·65 h^{-1}, respectively) obtained without preincubation of dough (Table 3).

Discussion

The measurement of bacterial-specific growth rate in sourdough, the key factor determining the competitiveness of individual species of LAB, is not a trivial task. Two different high throughput methods were used in this work. Using the IMC, the specific growth rates of the individual bacteria strains were measured in dough from power–time curves while the measurements in 'Bioscreen' were based on OD–time plots in flour extract or MRS broth.

The maximum specific growth rates of individual bacteria measured under external aerobic conditions in sourdough and those of flour extract or MRS broth were very different – the maximum specific growth rates of several sourdough bacteria strains in sourdough extract were only half of that measured in dough (Table 3), whereas in anaerobic conditions, the maximum growth rates determined by IMC in dough and by the 'Bioscreen' method in MRS or dough extract generally were comparable.

The growth of industrial sourdough bacteria (*Lact. helveticus* N92, *Lact. helveticus* E96, *Lact. vaginalis* N1113, *Lact. vaginalis* E1113 and *Lact. pontis* N131) appeared to be inhibited by oxygen. Their maximum growth rates measured in 'Bioscreen' mixed wells in aerobic conditions were significantly lower compared with the values obtained in anaerobic conditions or during microcalorimetric measurements. It can be suggested that during solid-state fermentation in industrial dough similarly to the fermentation in calorimetric vials, the sourdough

bacteria were protected form the inhibitory effect of oxygen by the dough matrix. The reversible sorption of oxygen by dough starch and gluten leading to low levels of dissolved oxygen in flour water slurry (Xu 2001) as well as the anti-oxidative effect of flour (Michalska *et al.* 2007; Zielinski *et al.* 2007) has been demonstrated. The later explains the growth of *Lact. vaginalis* and *Lact. panis* in aerobic conditions in flour extract but not in MRS.

The results suggested that the oxygen is one of the most important factors determining the growth performance and formation of consortium of bacteria in sourdough and that growth characteristics in liquid- and solid-state culture even with same nutritional composition may vary significantly. Therefore, it is important to measure the growth in the 'natural' environment. The plating-out method is laborious and because of the low colony-forming efficiency of rye sourdough bacteria might not work and indirect methods should be used. As show the results, the IMC is the good choice for characterization of growth of individual bacteria in rye sourdough.

The main problem with the IMC method was that the specific growth rate μ can be derived from power–time curves only in exponential growth phase during which both the growth yield and specific heat production rates should remain constant [see formula (5)]. In the nonexponential growth phase, the specific growth rate can be derived from power–time curves if the biomass yield Y_{XH} based on heat production remains constant[formula (4)]. As Y_{XH} probably decreases smoothly in the postexponential growth phase with decreasing pH, the real specific growth rates remain lower than those calculated according to formula (5). The calculation of specific growth rate from the 'Bioscreen' OD–time plots, as opposed to the IMC, was not restricted to exponential growth phase or constant growth yield [eqns (1–3) and (5)]. The main problem with the 'Bioscreen' measurements was that the μ of individual strains could not be directly measured in an opaque dough environment and there was need to assume that the growth characteristics of bacteria in flour extract or MRS are similar to those in sourdough.

Analysis of the power–time curves obtained for heterofermentative LAB revealed an intermediate decrease in heat flow before reaching heat flow maximum (Fig. 2). Such decrease was not observed in 'Bioscreen' experiments where OD increased continuously and μ after achieving its maximum decreased continuously for all strains studied (data not shown). The intermediate heat flow decrease in samples inoculated with heterofermentative LAB might be because of a change in stoichiometry of energy source utilization. During the first growth stage, fructose, which was forming because of the activity of indigenous flour enzymes (Table 2), was converted by heterofermentative bacteria into mannitol, acetate and lactate (Zaunmüller *et al.* 2006):

$$2\text{Fructose} + \text{glucose} \rightarrow 2\text{mannitol} + \text{acetate}^- + \text{CO}_2 + \text{lactate}^- + 2\text{H}^+ \qquad -605\,\text{J (2 mmol per acid)}$$

Based on simplified thermodynamic calculations which do not take into account biomass formation and based on the values of free energies of substrates and products formed (Goldberg and Alberty 2010), about −300 J per mmol of acid (lactate and acetate) would be formed. In the second growth stage, if fructose becomes a growth limiting factor, the bacteria might smoothly switch to a thermodynamically less favourable process, which is accompanied with ethanol production:

$$\text{Glucose} \rightarrow \text{lactate}^- + \text{ethanol} + \text{CO}_2 + \text{H}^+ \qquad -217\,\text{J mmol ATP}^{-1}\text{(or acid)}$$

This is reflected by sharp up to 30% decrease in heat flow (Fig. 2a,b) in the case of heterofermentative bacteria (Fig. 2c,d) after the initial stages of fermentation.
In case of homofermentative species, lactic acid was produced from glucose and according to thermodynamic data −100 kJ mol lactate^{-1} should be released:

$$\text{Glucose} \rightarrow 2\text{Lactate}^- + 2\text{H}^+ \qquad -200\,\text{J mmol}^{-1}\text{glucose}$$

The average experimental values obtained for homo- and heterofermentative LAB species were 89 ± 8 J mmol acid^{-1} and 167 ± 22 J mmol acid^{-1}, respectively. Those are lower than the values predicted above as the energy used for ATP formation (*c.* 36 J mmol^{-1}) was partly used for the biomass synthesis and was not released as a heat.

Using those accumulated heat yields as well as titration curve, it was possible to convert the power–time curves into specific growth rate to pH curves. Those curves showed that bacteria *Lact. helveticus* N92, *Lact. helveticus* E96, *Lact. casei* N726, *Lact. vaginalis* N1113, *Lact. vaginalis* E1113, *Lact. panis* N915 and *Lact. pontis* N131 isolated from the industrial sourdough had lower maximum growth rate values than the strains *Lact. curvatus* 0E12-11, *Lact. sakei* 0E12-10, *Lact. brevis* 0E12-37, *Leuc. citreum* 3N18-10 isolated from the spontaneously fermented laboratory sourdough. However, the industrial isolates showed higher growth rate at low pH values (Fig. 3). This could be explained by the difference of applied selection pressure and its duration in these two microbial consortia. The fast growing strains were dominating in the spontaneously started sourdough microbial population at least for the first 3 weeks after the start, whereas the titratable acidity of the spontaneous sourdough remained about twofold lower than that measured in the industrial

sourdough. The long-term selective pressure of decreasing pH in industrial sourdough resulted in domination of an acid-tolerant LAB population. In addition, the adaptation of microbial population to the semi-anaerobic conditions present in the industrial sourdough because of the high water content and high volumes has occurred resulting in the selection of the microbial consortiums unique to the current industrial sourdough cycle conditions.

Conclusions

Isothermal microcalorimetry in combination with chemical analysis is one of most promising high throughput methods for the comparative characterization of the performance of individual species of bacteria in sourdough. Measurements of the power–time curves of species of lactic acid bacteria in sourdough revealed that the LAB strains dominating in the industrial sourdough cycle had better acid tolerance but lower maximum growth rate and oxygen tolerance than species isolated from a 'fresh' sourdough cycle.

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References

Abd-Aziz, S., Gan Siew, H., Mohd Ali, H., Mohamed Ismail, A.K. and Noraini, S. (2008) Indirect method for quantification of cell biomass during solid-state fermentation of palm kernel cake based on protein content. *Asian J Scientific Res* **1**, 385–393.

Braissant, O., Wirz, D., Goepfert, B. and Daniels, A.U. (2010) Use of isothermal microcalorimetry to monitor microbial activities. *FEMS Microbiol Lett* **303**, 1–8.

Corsetti, A. and Setanni, L. (2007) Lactobacilli in sourdough fermentation. *Food Res Int* **40**, 539–558.

De San, N., Denis, O., Gasasira, M.-F., De Mendonca, R., Nonhoff, C. and Struelens, J. (2007) Controlled evaluation of the IDI-MRSA assay for detection of colonization by Methicillin-Resistant *Staphylococcus aureus* in diverse mucocutaneous specimens. *J Clin Microbiol* **45**, 1098–1101.

De Vuyst, L. and Neysens, P. (2005) The sourdough microflora: biodiversity and metabolic interactions. *Trends Food Sci Technol* **16**, 43–56.

De Vuyst, L. and Vancanneyt, M. (2007) Biodiversity and identification of sourdough lactic acid bacteria. *Food Microbiol* **24**, 120–127.

De Vuyst, L., Vrancken, G., Ravyts, F., Rimaux, T. and Weckx, S. (2009) Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota. *Food Microbiol* **26**(7), 666–675.

Fang, H. and Hedin, G. (2006) Use of Cefoxitin-based selective broth for improved detection of methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol* **44**, 592–594.

Forrest, W.W. and Walker, D.J. (1963) Calorimetric measurements of energy of maintenance of *Streptococcus faecalis*. *Biochem Biophys Res Commun* **13**, 217–222.

Goldberg, R.N. and Alberty, R.A. (2010) Standard transformed Gibbs energies of formation for biochemical reactants. In *CRC Handbook of Chemistry and Physics* ed. Lide, D.R. Chapter 7, pp. 10–12. Florida: CRC Press.

Gustafsson, L. (1991) Microbiological calorimetry. *Thermochim Acta* **193**, 145–171.

Häggman, M. and Salovaara, H. (2008) Microbial re-inoculation reveals differences in the leavening power of sourdough yeast strains. *Lebensm Wiss Technol* **41**, 148–154.

Hammes, W.P., Brandt, M.J., Francis, K.L., Rosenheim, J., Seitter, M.F. and Vogelmann, S.A. (2005) Microbial ecology of cereal fermentation. *Int J Food Microbiol* **16**, 4–11.

James, M.A. (1987) *Thermal and Energetic Studies of Cellular Biological Systems*. pp. 221. Bristol: Wright.

Joye, I.J., Lagrain, B. and Delcour, J.A. (2009) Use of chemical redox agents and exogenous enzymes to modify the protein network during breadmaking. *J Cereal Sci*, **50**, 11–21.

Kabanova, N., Kazarjan, A., Stulova, I. and Vilu, R. (2009) Microcalorimetric study of growth of *Lactococus lactis* IL1403 at different glucose concentrations in broth. *Thermochim Acta* **496**, 87–92.

Katina, K., Sauri, M., Alakomi, H.-L. and Mattila-Sandholm, T. (2002) Potential of lactic acid basteria to inhibit rope spoilage in wheat sourdough bread. *Lebensm Wiss Technol* **35**, 38–45.

Menert, A., M. Liiders, M., Kurissoo, T. and Vilu, R. (2001) Microcalorimetric monitoring of anaerobic digestion processes. *J Therm Anal Calorim* **64**, 281–291.

Michalska, A., Ceglinska, A. and Zielinski, H. (2007) Bioactive compounds in rye flours with different extraction rates. *Eur Food Res Technol* **225**, 545–551.

Oriol, E., Conteras, R. and Raimbault, M. (1987) Use of microcalorimetry for monitoring the solid state culture of aspergillus niger. *Biotechnol Tech* **1**, 79–84.

Rosenquist, H. and Hansen, A. (2000) The microbial stability of two bakery sourdoughs made from conventionally and organically grown rye. *Food Microbiol* **17**, 241–250.

Terebiznik, M.R. and Pilosof, A.M.R. (1999) Biomass estimation in solid state fermentation by modeling dry matter weight loss. *Biotechnol Tech* **13**, 215–219.

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

Vandenhove, H. (1998) Microcalorimetric characterization of bacterial inocula. In *Advanced Instrumentation, Data Interpretation, and Control of Biotechnological Processes* ed. Impe, J.F., Vanrolleghem, P.A. and Iserentant, D.M. pp. 121–158 Netherlands: Springer.

Vera, A., Rigobello, V. and Demarigny, Y. (2009) Comparative study of culture media used for sourdough lactobacilli. *Food Microbiol* **26**, 728–733.

Von Ah, U., Wirz, D. and Daniels, A.U. (2008) Rapid MSSA-MRSA differentiation and MIC determinations by isothermal microcalorimetry. *J Clin Microbiol* **46**, 2083–2087.

Wadsö, L. (1997) Isothermal microcalorimetry near ambient temperature: an overview and discussion. *Thermochim Acta* **294**, 1–11.

Wadsö, I. (2002) Isothermal microcalorimetry in applied biology. *Thermochim Acta* **394**, 305–311.

Wadsö, L. and Galindo, F.G. (2009) Isothermal calorimetry for biological applications in food science and technology. *Food Control* **20**, 956–961.

Wadsö, L., Li, Y. and Bjurman, J. (2004) Measurements on two mould fungi with a calorespirometric method. *Thermochim Acta* **422**, 63–68.

Xu, F. (2001) Adsorption of oxygen gas by hydrated wheat flour. *Lebensm Wiss Technol* **34**, 66–70.

Yi, L., Xi, L., Songsheng, Q. and Ping, S.I. (2000) Microcalorimetric investigation of the toxic action of Cd^{2+} on *Rhizopus nigricans* growth. *J Biochem Biophys Methods* **45**, 231–239.

Zaunmüller, T., Eichert, M., Richter, H. and Unden, G. (2006) Variations in the energy metabolism of biotechnologically relevant heterofermentative lactic acid bacteria during growth on sugars and organic acids. *Appl Microbiol Biotechnol* **72**, 421–429.

Zielinski, H., Ceglinska, A. and Michalska, A. (2007) Antioxidant contents and properties as quality indies of rye cultivars. *Food Chem* **104**, 980–988.

PUBLICATION II

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Analysis of volatile compounds produced by different species of Lactobacilli in rye sourdough using multiple headspace extraction

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Original article

Analysis of volatile compounds produced by different species of lactobacilli in rye sourdough using multiple headspace extraction

Kristel Kaseleht,[1,2]* Toomas Paalme,[1,2] Anna Mihhalevski[1,2] & Inga Sarand[1,2]

1 Competence Center of Food and Fermentation Technologies, Akadeemia tee 15b, Tallinn 12618, Estonia
2 Tallinn University of Technology, Ehitajate tee 5, Tallinn 12618, Estonia

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Summary Profiles of volatile organic compound (VOC) produced by nine individual lactic acid bacteria (LAB) during rye sourdough fermentation were compared by automated SPME and GC/MS-Tof. The dough samples were inoculated with individual strains, placed inside the headspace vials and incubated during next 24 h. The production or loss of VOC-s was followed by adsorbing volatiles onto 85-m Car/PDMS fibre in every 4 h. Volatile profiles differed among LAB species and divided LAB into two main groups – hetero- and homofermentative. Hetrofermentative LAB (*Lactobacillus brevis*; *Leuconostoc citreum*; *Lactobacillus vaginalis*, *Lactobacillus panis*) showed high production of acetic acid, CO_2, ethanol, ethylacetate, producing also hexyl acetate, ethyl hexanoate and isopentyl acetate. Whereas homofermentative LAB species (*Lactobacillus helveticus*; *Lactobacillus casei; Lactobacillus sakei*; *Lactobacillus curvatus*) produced a considerable amount of 2,3-butanedione. Production of L-leucine methyl ester was unique for *Lb. sakei*, *Lb. casei* and *Lb. curvatus* strains. *Lb. helveticus* was the only LAB that produced benzaldehyde.

Keywords Lactic acid bacteria, multiple headspace extraction, rye sourdough, solid-phase microextraction, volatile compounds.

Introduction

Sourdough is essential to rye bread by improving bread quality in terms of delaying staling, improving taste, texture, aroma and generally increasing shelf life (Plessas *et al*., 2008). As rye does not contain enough gluten, the structure of rye bread depends on the starch in the rye flour, as well as other carbohydrates known as pentosans (Wing & Scott, 1999).

Rye bread flavour is influenced by the recipe, processing conditions and its starter culture composition. Lactic acid bacteria (LAB) are the main part of sourdough starter. Although LAB are mostly responsible for the sourdough acidification, they also liberate aroma precursors. It is, however, not fully clear to what extent different LAB contribute to flavour formation.

The generation of odorants occures in sourdough mainly because of enzymatic and microbial processes during sourdough fermentation; however, some compounds are already present in the rye flour (Kirchoff & Schieberle, 2002).

Homofermentative LAB present in sourdough are able to convert hexoses almost completely into lactic acid ($>85\%$), whereas heterofermentative LAB degrade hexoses into lactic acid, acetic acid, ethanol and CO_2 (Hansen & Schieberle, 2005). In addition, LAB liberate aroma precursors, such as free amino acids, which concentrations increase significantly during flour hydration (Mihhalevski *et al*., 2010) and sourdough fermentation (Hansen & Schieberle, 2005). The key degradation reaction of amino acids during dough fermentation is the *Ehrlich* pathway leading to formation of aldehydes or the corresponding alcohols (Hansen & Schieberle, 2005). The composition of volatile fraction of rye sourdough has been found to consist of acetic acid, ethanol and volatile compounds. In addition, the increased amounts of esters, such as acetates, propionates, hexanoates, lactates and octanoates, have been reported; also, (*E*)-2-butenal, (*Z*)-3-hexenal, nonanal and heptadienal were characterised as sourdough constituents (Kirchoff & Schieberle, 2002). The number of aldehydes was lower in sourdoughs fermented by heterofermentative LAB (Kirchoff & Schieberle, 2002). Whereas the content of ethyl acetate as well as hexyl acetate has been shown to be higher in rye sourdoughs fermented with heterofermentative LAB compared with sourdoughs fermented with homofermentative LAB (Lund *et al*., 1989).

The aim of this work was to study the impact of different LAB strains on volatile organic compounds

*Correspondent: Fax: +372 6 408 282;
e-mail: kkrissu@gmail.com

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formation in sourdough and its significance on rye bread aroma development using multiple headspace extraction (MHE). MHE is originally a technique for quantifying analytes in solid samples, which avoids matrix effect (implying several consecutive extractions from the same sample until no volatiles are present) (Ezquerro *et al*., 2003), but is used for monitoring fermentation dynamics in this study.

Although volatile compounds in rye sourdough have been studied previously (Kirchoff & Schieberle, 2002; Hansen & Schieberle, 2005), no data are available on the dynamics of volatile compound patterns during dough fermentation with single LAB strains. The 10 kGy irradiated flour was used to prevent the influence of indigenous flour LAB on aroma formation. A 24-h fermentation at 24 °C without addition of yeasts was used to simulate rye sourdough fermentation conditions used in the local bakery.

Materials and methods

Microorganisms

The lactobacilli strains *Lactobacillus helveticus* N92, *Lactobacillus helveticus* E96, *Lactobacillus casei* N726, *Lactobacillus vaginalis* N1113, *Lactobacillus panis* N915 were isolated from industrial sourdough. *Lactobacillus curvatus* 0E12-11, *Lactobacillus sakei* 0E12-10, *Leuconostoc citreum* 3N18-10, *Lactobacillus brevis* 0E12-37 were isolated from spontaneously fermented rye flour. The bacteria were isolated and identified based on 16S r-RNA sequence (Viiard *et al*., unpublished) essentially as described by Van der Meulen *et al*. (2007). The strains were maintained in stock as 25% glycerol cultures at −80 °C and re-cultivated anaerobically in De Man, Rogos and Sharpe (MRS) broth (Lab M, Bury, UK) 48 h at 30 °C for the dough fermentation experiments (Mihhalevski *et al*., 2010).

Chemicals and materials

Rye flour was purchased from Tartu Veski (Tartu, Estonia) and irradiated at 10 kGy using dosimetric system GEX WinDose (Gex Corporation, Centennial, CO, USA). Helium, purity 5.5 (AGA Eesti AS, Tallinn Estonia), was used as the carrier gas in the GC system. Silica SPME fibre with 85-μm Car/PDMS coating (chosen according to Kaseleht *et al*., 2010) was obtained from Supelco (Bellefonte, PA, USA). Fibre was conditioned according to the manufacturer's recommendations prior to its first use. Clear glass SPME crimp vials (20 mL) with the polytetrauoroethylene (PTFE)/silicone septa (20 mm) were purchased from Supelco. MilliQ water (Millipore Corp., Molsheim, France) was used whenever samples were prepared.

Sample preparation

Bacterial suspension ($1–5 \times 10^7$ cfu mL^{-1}) in 0.5% NaCl was mixed with sterile rye flour (1:1) and mixed for 15 min using a model 400 circulator stomacher (Seward Ltd., Worthing, UK). For VOC analysis, 1 g of sourdough was weighed in two parallels into 20 mL headspace vial and sealed with the crimp cap. Noninoculated sterile dough was used as control. First, VOC analysis of dough was performed immediately after sealing the vial and placing it in the autosampler tray (about 15 min after flour hydratation and inoculation), after which the sample vials were held sealed and incubated at 24 °C on the sample tray for MHE. The VOC was extracted by SPME technique from the vials at 4, 9, 14, 20 and 24 h to monitor the changes in sourdough VOCs during growth of nine different LAB and a noninoculated control.

GC/MS analysis

To analyse sourdough volatile compounds in the sample vials, the SPME technique was adopted with an autosampler CombiPAL (CTC Analytics, Zwingen, Switzerland) and GC/MS-Tof (Agilent Technologies Inc., Santa Clara, CA, USA and Waters, Manchester, UK). The volatiles adsorbed onto the SPME fibre during 20-min extraction were thermally desorbed 10 min at 260 °C in the injection port of an Agilent 6890 gas chromatograph equipped with a DB-5MS column (J&W Scientific, Folsom, CA, USA) with a 0.25 μm film thickness, 30 m length and 0.32 mm inner diameter (i.d.). Helium (1.7 mL min^{-1}) was used as the carrier gas, and the injector was in splitless mode for 10 min using an inlet liner of 0.75 mm i.d. The detector temperature was 200 °C, and for ionisation, electron energy of 70 eV was used. The column temperature was initially maintained at 35 °C for 3 min after injection before increasing to 155 °C at a rate of 7 °C min^{-1} and to 280 °C at a rate of 45 °C min^{-1} with a total run time of 22.92 min. Volatile compounds were tentatively identified by comparing their spectra and retention indices with those present in the NIST05 library (NIST, Gaithersburg, MD, USA) using MassLynx and ChromaLynx software (Waters). For intensity analysis, the peak areas were integrated and compared using MassLynx software.

Results and discussion

Volatile formation in noninoculated rye sourdough

Comparison of SPME chromatograms of the noninoculated dough prepared from 10 kGy rye flour demonstrated that chromatographic pattern changed during 24-h incubation (Fig. 1a, *peaks 1–34*, and b, *peaks 1–34*

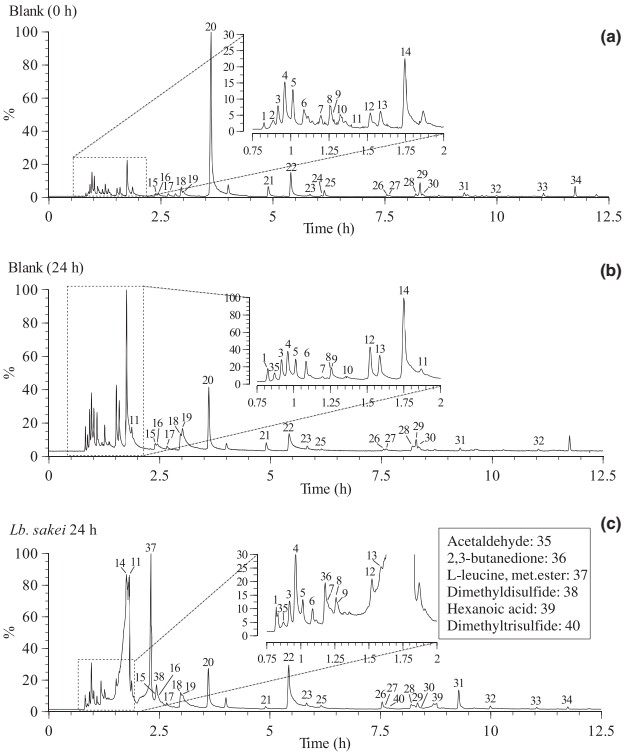

Figure 1 Chromatograms of 10 kGy irradiated dough at 0 and 24 hour (a and b). Chromatogram of 10 kGy irradiated dough after inoculation with Lb. sakei after 24 hours (c). Peak numbers correspond to the compounds in Table 1.

and peak 35). The change was the result of biological and chemical processes in sourdough and removal of volatiles by SPME fibre. Although the compounds were repeatedly taken out with the fibre, the concentration of several compounds like 3-methylbutanal (*peak 12*), 2-methylbutanal (*13*), carbon dioxide (*1*), acetaldehyde, ethanol (*3*), propanal (*4*), 2-methylpropanol (*5*), 2-butanone (*7*), ethyl acetate (*9*), acetic acid (*11*), 1-penten-3-ol (*14*), (Z)-2-penten-1-ol (*19*) increased in the vials during incubation. Compounds that decreased during 24-h incubation were 2-propenal (*2*), 2(3)-methylfuran (*8*), trichloromethane (*10*), 3-methylbutanol (*15*), 2-methylbutanol (*16*), (E)-2-pentenal (*17*), 1-pentanol (*18*), hexanal (*20*), 2-hexenal (*21*), 1- hexanol (*22*), 2-heptanone (*23*), (Z)-4-heptenal (*24*), heptanal (*25*), (E)-2-heptenal (*26*), benzaldehyde (*27*), 1-octen-3-ol (*28*), 2-pentylfuran (*29*), 2-octanone (*30*), 2-octenal (*32*), nonanal (*33*), 2-nonenal (*34*). The production of those compounds was less than the amount transferred out with the fibre during repeated sampling.

Volatile compound formation in dough inoculated by individual LAB strains

Analysis of volatile peak intensities (Total Ion Count, TIC) at different time points of SPME extraction during 24-h fermentation of rye dough inoculated with nine different LAB was carried out. Both homofermentative (*Lb. sakei, Lb. casei, Lb. curvatus, Lb. helveticus E96 and Lb. helveticus N92*) and heterofermentative (*Lb. brevis, Ln. citreum, Lb. panis, Lb. vaginalis*) LAB strains were used for inoculation. To determine the volatile compound production profiles of studied LAB, the 10 kGy noninoculated dough incubation profiles were subtracted from the profiles obtained for correspondent inoculated dough samples. All the compounds tentatively identified in dough are listed in Table 1.

Inoculation of dough significantly affected the SPME volatile profiles in fermentation vials, for example 24-h *Lb.sakei* (Fig. 1c, *peaks 1–34* and *peaks 35–40*) vs. 24-h blank (Fig. 1b). Appearance of hexanoic acid, by-product of fatty acid biosynthesis, on chromatogram

Table 1 Profiles of volatile compounds formation during growth of individual bacteria in dough. 'Peak' presents the peak number in Fig. 1, 'Control' is noninoculated control prepared from 10 kGy irradiated rye flour. Compounds were either not detected (0), decreasing (−) or increasing (+) during growth phase of corresponding lactic acid bacteria

			Heterofermentative				Homefermentative				
Compound	**Peak**	**Control**	***Lb. brevis***	***Ln. citreum***	***Lb. panis***	***Lb. vaginalis***	***Lb. sakei***	***Lb.curvatus***	***Lb.casei***	***Lb.helv.E96***	***Lb.helv.N92***
Carbon dioxide	1	+	++	++	++	++	++	++	+	+	+
2-propenal	2	−	0	0	−	−	0	0	0	−	−
2-propen-1-ol		0	−	0	−	+	0	0	0	0	0
Acetaldehyde	35	+	− −	− −	− −	− −	+	+	−	+	+
Ethanol	3	+	+++	+++	+++	+++	+	+	+	+	+
Acetic acid	11	+	++++	++++	+++	+++	+++	+++	+	++	+
Ethyl acetate	9	+	+++	+++	+++	++	−	−	−	−	−
Propanal	4	+	−	− −	− −	− −	+	+	+	+	+
2-methylpropanal	6	+	−	− −	− −	−	+	+	−	+	+
2-methylpropanol	5	+	+	+	+	+	+	+	+	+	+
3-methylbutanal	12	++	− −	− −	− −	− −	+	−	−	−	+
3-methylbutanol	15	− −	+	+	+	+	+	+	+	+	+
2-butanone	7	+	−	−	−	−	+	+	+	+	+
2-methylbutanal	13	++	− −	− −	− −	− −	−	−	−	−	+
2-methylbutanol	16	− −	+	+	+	+	+	+	+	+	+
2-pentenal (E)	17	−	0	−	−	−	+	+	+	+	+
1-pentanol	18	−	+	+	+	+	+	+	+	+	+
1-penten-3-ol	14	+	−	+	+	−	+	+	+	+	+
2-penten-1-ol (Z)	19	+	+	+	+	+	+	+	+	+	+
Isopentyl acetate		0	+	+	+	+	0	0	0	0	0
Hexanal	20	− − −	− − −	− − −	− − −	− − −	−	− −	− − −	− −	− −
1-hexanol	22	− − −	++	++	++	++	+	+	+	+	−
2-hexenal	21	− −	− −	− −	0	− −	−	−	−	+	−
Hexanoic acid	39	0	++	+++	++	++	++	+++	++	++	++
Hexyl acetate		0	++	++	+	+	0	0	0	0	0
Ethyl hexanoate		0	+	+	+	0	0	0	0	0	0
Heptanal	25	− − −	− − −	− − −	0	− − −	−	−	−	−	−
4-heptenal (Z)	24	− − −	− − −	− − −	0	− − −	− − −	−	− − −	− − −	− − −
2-heptenal (E)	26	− −	+	−	+	−	++	+	+	+	+
2-heptanone	23	−	−	−	+	+	+	+	+	+	+
2-octanone	30	− − −	−	−	−	−	−	−	−	−	−
2-octenal	32	− − −	0	− − −	0	− − −	+	+	− − −	+	− − −
1-octen-3-ol	28	−	+	+	+	+	+	+	+	−	− −
Nonanal	33	− −	−	−	−	− −	+	+	+	+	+
2-nonenal	34	− − −	− − −	− − −	0	− − −	−	−	− − −	− − −	−
2(3)-methylfuran	8	−	−	−	−	−	−	−	−	−	−
2-pentylfuran	29	− − −	+	+	+	+	+	+	+	+	+
Trichloromethane	10	−	0	0	0	−	0	−	0	0	−
Benzaldehyde	27	− −	− −	− −	0	− −	−	−	− −	++	++
Dimethyldisulfide	38	0	++	+	+	+	++	++	+	+	+
Dimethyltrisulfide	40	0	+	0	0	0	+	+	0	0	0
L-leucine,met.ester	37	0	0	0	+	0	+++	+++	++	0	0
2,3-butanedione	36	0	0	0	0	0	+	++	++++	+	+
Unknown 9.27	31	− −	+	++	−	−	++	++	++	++	+

was used as an indicator of growth start of LAB. As growth rates of the inoculated LAB strains differ, appearing of hexanoic acid on chromatogram was used as starting point for comparative characterisation of volatile compound formation during growth of individual species, for example during 14–19 h after inoculation for *Lb. panis* and 19–26 h for *Lb. helveticus N92* (Fig. 2).

The results obtained show that hexanoic acid, 1-hexanol, 1-pentanol, 2- and 3-methylbutanol,

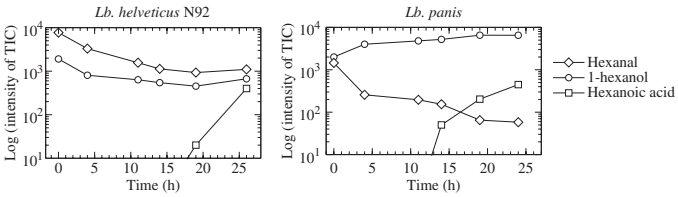

Figure 2 Intensities (total ion count) of hexanal, 1-hexanol and hexanoic acid during 24 hours in homofermentative *Lb. helveticus* N92 and heterofermentative *Lb. panis*.

2-pentylfuran and dimethyldisulfide were produced and 2- and 3- methylbutanal consumed during growth of all LAB species in sourdough (Table 1).

The production of alchohols suggests that so-called *Ehrlich* aldehydes, including 2- and 3-methylbutanal, originally present in the flour and additionally forming during dough incubation were reduced by LAB into corresponding alcohols: 2- and 3-methylbutanol (peaks *15* and *16*), respectively. Taking into account also the formation of hexanol and heptanol, it can be suggested that dough environment with growth of LAB becomes reducing (Vermeulen *et al*. 2007). Most probably, LAB use the aldehydes present in dough as electron acceptors in alcohol dehydrogenase reaction (EC 1.1.1.1) to oxidise NADH produced in excess during the growth of LAB. The ability to reduce aldehydes was more pronounced for heterofermentative species than for homofermentative.

Heterofermentive species

The aroma profiles of different species in sourdough correspond well to division of LAB to homo- and heterofermentative species. As expected, the inoculation of dough with heterofermentative species using phosphoketolase pathway resulted in remarkable CO_2, ethanol and acetic acid production. Those compounds were produced also by homofermentative species but at much lower rate.

While reduction in aldehydes (2- and 3-methylbutanal, 2-hexenal, (E)-2-heptenal, hexanal, heptanal and 2-octenal) was more strongly expressed in hetero- than homofermentative species, the reduction in acetaldehyde, propanal, 2-methylpropanal, 2-butanone into corresponding alcohols was observed only in case of heterofermentative species. The differences were probably owing to the higher alcohol dehydrogenase activity and/or higher surplus of reductive power (NADH) during the growth of heterofermentative species (Fig. 3).

Homofermentative species, instead of degrading, produced (E)-2-pentenal and nonanal and also produced less 1-hexanol than heterofermentative species.

Production of esters like ethyl acetate, isopentyl acetate, hexyl acetate, ethyl hexanoate was also specific only to heterofermentative species. That is because of

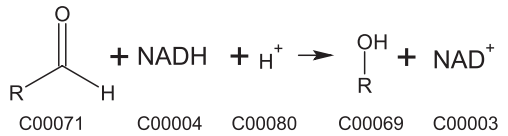

Figure 3 Reaction pathway of alcohols derived from aldehydes.

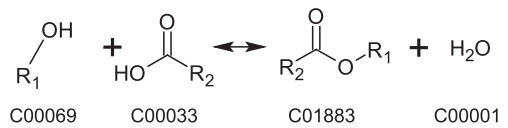

Figure 4 Reaction pathway for esters from alcohol and organic acid.

the higher production of ethanol and acetate by heterofermentative species (Fig. 4).

Our results show that there were no significant differences in volatile compound formation profiles within heterofermentative species studied.

Homofermentive species

All homofermentative lactobacilli produced 2,3-butanedione (particularly *Lb. casei*), which could not be seen in case of heterofermentative species. This was also observed by Hansen & Schieberle, 2005; who stated that the content of diacetyl was higher in sourdoughs manufactured with homofermentative compared with heterofermentative cultures and was also higher in the corresponding breads. Probably, the heterofermetative bacteria lack pathway for diacetyl synthesis, or/and the redox potential is too high to allow oxidation of acetoin (Fig. 5).

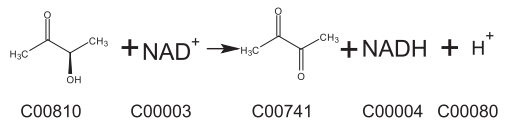

Figure 5 Reaction pathway of diacetyl from acetoin.

Lb. sakei, *Lb. curvatus* and *Lb. casei* were producing L-leucine methyl ester; however, a very small peak was also found in heterofermentative *Lb. panis*.

L. sakei and *L. curvatus* strains were rather similar. They were isolated from the first stages of spontaneous fermentation of sourdough. These species had high maximum specific growth rate in rye dough (0.8–0.9 h^{-1}) (Mihhalevski *et al*., 2010) and were unique in producing considerable amount of L-leucine methyl ester. The compound is probably derived by esterification of leucine and methanol. The reasons for the formation of L-leucine methyl ester are not clear. Also, significant acetate production was observed in those species compared with other homofermentative LAB.

Lb. helveticus E96 and *Lb. helveticus* N92 were dominating strains in repeated cycle of industrial rye sourdough in local bakery. Compared to other species studied the *Lb. helveticus* was unique in producing benzaldehyde. Benzaldehyde has been reported to be produced from phenylalanine in *Lb. plantarum* (Groot & de Bont, 1998). In addition, different from all the other dough studied, 1-hexanol decreased in dough inoculated with *Lb. helveticus* N92 strain. This might be explained by the low extent of reduction in 1-hexanal to 1-hexanol compared with other strains.

Significant formation of L-leucine methyl ester was observed for dough inoculated with the *Lb. casei* strain. Also, the amount of synthesised 2,3-butanedione was the largest among homofermentative LAB strains studied.

Multiple headspace extraction of volatiles

The MHE/SPME method presents certain drawbacks, which limits its use for quantitative purposes. In case of sourdough volatile analysis, MHE technique could be used for quantification after the fermentation is stopped or samples are fixed. Doing that we can loose the main advantages of the technique: minimal processing of samples and high throughput as well as ability to follow dynamics of volatile formation during fermentation in single vial.

During MHE of dough samples, the amount VOC detected is the result of two processes: volatile formation and headspace extraction. If the amount of extracted volatile (GC peak area) remains constant in two consequent measurements, it can be expected that production rate between those two points is equal to amount of extracted compound divided by time interval between extractions. For different fermentations and compounds, this point in different growth phases at different biomass concentrations might be different. It makes thus quantitative comparison of production rates of different volatiles in different species difficult, and process quantification using SPME method would be possible using labelled internal standards.

In this study, the method conducted with automated SPME-GC/MS was used for semi-quantitative purposes and to identify the volatile production profiles during growth of different species of sourdough bacteria in their natural environment and relate them to corresponding chemical and biochemical transformations in sourdough. The culture conditions during sourdough fermentations in headspace vials were similar as possible to those used in the bakery. The temperature used was 24 °C, although higher fermentation temperatures are used in many bakeries. All those temperatures remain probably in range of Arrhenius plot, which affect mainly the specific growth rate but not other physiological properties of the culture (Adamberg *et al*., 2003). To follow the dynamics of the process, the fermentation was started from lower cell densities and higher pH than in industry. Low pH can cause the acid stress and affect the both volatile production rate and extraction coefficient. Next subject to study would be aroma formation in mixed cultures to answer the question if the aroma profiles in mixed culture differ from the sum of individual cultures.

The results of this study demonstrated that multiple extractions from the same sample vial using SPME fibre is a perspective high throughput method for characterisation of volatile formation profiles of microorganisms, in particular during fermentation in solid phase, like dough. That is particularly because of the automatisation that allows analysing several different samples in parallel and minimises human factor by allowing precise sampling and less fibre damage.

Present work gives good qualitative information about volatile formation and conversion processes taking place in the headspace vial during dough incubation and enables to see the differences between different strains of lactobacilli. How the aroma profile of sourdough bacteria affects the final sensory properties of bread is the goal for another study. Multiple headspace extraction in combination with sensory analysis is certainly perspective approach in such studies.

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References

Adamberg, K., Kask, S., Laht, T.M. & Paalme, T. (2003). The effect of temperature and pH on the growth of lactic acid bacteria: a pH-auxostat study. *International Journal of Food Microbiol*, **85**, 171–183.

Ezquerro, Ó., Pons, B. & Tena, M.T. (2003). Evaluation of multiple solid-phase microextraction as a technique to remove the matrix effect in packaging analysis for determination of volatile organic compounds. *Journal of Chromatography A*, **1020**, 189–197.

Groot, M.N. & de Bont, J.A. (1998). Conversion of phenylalanine to benzaldehyde initiated by aminotransferase in *Lactobacillus plantarum*. *Applied and Environmental Microbiology*, **64**, 3009–3013.
Hansen, A. & Schieberle, P. (2005). Generation of aroma compounds during sourdough fermentation: applied and fundamental aspects. *Trends in Food Science & Technology*, **16**, 85–94.
Kaseleht, K., Leitner, E. & Paalme, T. (2010). Determining aroma-active compounds in Kama flour using SPME-GC/MS and GC–olfactometry. *Flavour and Fragrance Journal*, **26**, 122–128.
Kirchoff, E. & Schieberle, P. (2002). Quantitation of odor-active compounds in rye flour and rye sourdough using stable isotope dilution assays. *Journal of Agricultural and Food Chemistry*, **50**, 5378–5385.
Lund, B., Hansen, A. & Lewis, M.J. (1989). The influence of dough yield on acidification and production of volatiles in sourdoughs. *LWT – Food Science and Technology*, **22**, 150–153.
Mihhalevski, A., Sarand, I., Viiard, E., Salumets, A. & Paalme, T. (2010). Growth characterization of individual rye sourdough bacteria by isothermal microcalorimetry. *Journal of Applied Microbiology*, **110**, 529–540.
Plessas, S., Bekatorou, A., Gallanagh, J., Nigam, P., Koutinas, A.A. & Psarianos, C. (2008). Evolution of aroma volatiles during storage of sourdough breads made by mixed cultures of Kluyveromyces marxianus and *Lactobacillus delbrueckii* ssp. *bulgaricus* or *Lactobacillus helveticus*. *Food Chemistry*, **107**, 883–889.
Van der Meulen, R., Scheirlinck, I., Van Schoor, A. *et al.* (2007). Population dynamic and metabolite target analysis of lactic acid bacteria during laboratory fermentations of wheat and spelt sourdoughs. *Applied and Environmental Microbiology*, **73**, 4741–4750.
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. *Journal of Cereal Science*, **45**, 78–87.
Wing, D. & Scott, A. (1999). *The Bread Builders*. Pp. 255. Vermont, USA: Chelsea Green Publishing.

PUBLICATION III

Viiard E, Mihhalevski A, Rühka T, Paalme T, Sarand I

Evaluation of the microbial community in industrial rye sourdough upon continuous backslopping propagation revealed *Lactobacillus helveticus* as the dominant species

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Evaluation of the microbial community in industrial rye sourdough upon continuous back-slopping propagation revealed *Lactobacillus helveticus* as the dominant species

Ene Viiard [a, b], Anna Mihhalevski [a, b], Tiina Rühka [c], Toomas Paalme [a, b], Inga Sarand [a, b, d *]

[a] Competence Center of Food and Fermentation Technologies, Akadeemia tee 15 A, 12618, Tallinn, Estonia

[b] Department of Food Processing, Tallinn University of Technology, Ehitajate tee 5, 19086, Tallinn, Estonia

[c] Leibur Ltd., Kadaka tee 76a, 12618, Tallinn, Estonia

[d] Department of Gene Technology, Tallinn University of Technology, Akadeemia tee 15, 12618, Tallinn, Estonia

[*] Corresponding author. Tel.: +372 6408200; fax: +372 6408282

E-mail address: inga@tftak.eu

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Abstract

Aims

To assess the structure and stability of a dominant lactic acid bacteria (LAB) population during the propagation of rye sourdough in an industrial semi-fluid production over a period of seven months.

Methods and Results

The sourdough was started from a six years old freeze-dried sourdough originating from the same bakery. A unique microbial consortium consisting mainly from bacteria belonging to species *Lactobacillus helveticus*, *Lactobacillus panis* and *Lactobacillus pontis* was identified based on culture dependent (Rep-PCR) and culture independent (denaturing gradient gel electrophoresis (DGGE)) methods. Three of the isolated *L. helveticus* strains showed remarkable adaptation to the sourdough conditions. They differed from the type strain by the ability to ferment compounds specific to plant material, like salicin, cellobiose and sucrose, but did not ferment lactose.

Conclusion

We showed remarkable stability of a LAB consortium in rye sourdough started from lyophilized sourdough and propagated in a large bakery for seven months. *L. helveticus* was detected as the dominant species in the consortium and was shown to be metabolically adapted to the sourdough environment.

Significance and Impact of study

The use of an established and adapted microbial consortium as a starter is a good alternative to commercial starter strains.

Running headline

LAB of industrial rye sourdough

Keywords

Rye sourdough; Lactic acid bacteria; *Lactobacillus helveticus*; industrial sourdough; back-slopping

1. Introduction

The taste, aroma and texture of sourdough breads greatly depend on starter cultures used for flour fermentation (Salim-ur-Rehman et al., 2006; Corsetti and Settanni, 2007). Rye sourdough cultures are mainly lactic acid bacteria (LAB) belonging to both homo- and heterofermentative species. So far the composition of microbial communities of rye sourdoughs used in different countries has been characterized mainly based on phenotypical traits (reviewed Hansen, 2004; De Vuyst and Neysens, 2005). *Lactobacillus brevis*, *Lactobacillus plantarum* and *Lactobacillus sanfranciscensis* are the species that have most frequently been isolated from rye sourdoughs. In addition *Lactobacillus amylovorus*, *Lactobacillus panis*, *Lactobacillus reuteri* (Rosenquist and Hansen, 2000) and *Lactobacillus fermentum* (Weckx et al., 2010) have been reported as dominating species in some rye sourdough consortia.

Several factors, including process parameters, production environment and type of flour, can affect the microbial composition of sourdoughs (De Vuyst and Neysens, 2005; Scheirlinck et al., 2007). Spontaneous fermentation of flour is commonly used to make sourdough in traditional bread making. Microbial consortia with remarkably high stability develop in such sourdoughs during continuous propagation. This kind of associations may endure propagation in the form of back-slopping for years, in spite of non-aseptic conditions (De Vuyst and

Neysens, 2005). According to Van der Meulen and co-authors (2007) it may take as short as ten days to establish a stable LAB consortium, but commonly it takes much longer.

The tendency in the manufacture of sourdough breads is to initiate fermentation by adding defined commercial starter cultures with specific properties (De Vuyst and Neysens, 2005). Unfortunately, commercial strains are often not competitive with endogenous microflora entering the sourdough back-slopping process from flour and the environment of the bakery (Meroth et al., 2003; Siragusa et al., 2009). Consequently frequent renewing of the sourdough cycling process is required. In our work we determined the composition of a lyophilized rye sourdough and monitored the dynamics of the microbial population in an industrial sourdough made from the lyophilized starter. The lyophilized sourdough aliquots had been prepared from sourdough used for bread making in the same bakery six years ago. The microbial community was monitored during seven months of daily propagation using both culture dependent and independent approaches.

2. Materials and Methods

2.1. Propagation and sampling of the sourdough

The sourdough was propagated in a large bakery (800 kg of sourdough) in Estonia. The lyophilized starter used for inoculation of the new sourdough was prepared from the sourdough of the same bakery six years before. It was stored at -20^{o}C in hermetically closed packages. The renewal of the industrial sourdough cycle was started by mixing lyophilized starter with rye flour and tap water in ratio 10:36:54 and incubating for 24 h at 32^{o}C. The following four back-slopping renewals were performed in the same ratio after 19, 12, 12 and 10 hours of fermentation. Routine industrial back-slopping was carried out with the same mother sponge, rye flour and water ratio 10:36:54 (sourdough yield 250 kg dough per 100 kg

flour) and fermentation at 32°C for 10 hours. Samples for chemical and microbiological analysis were collected at the end of fermentations.

2.2. Determination of pH and total titratable acidity

To measure pH and total titratable acidity (TTA) of fermented sourdough five grams of sample was suspended in 50 ml of distilled water. pH and TTA were measured with Food and Beverage Analyzer D22 (Mettler-Toledo International Inc., USA). The pH value was recorded and the acidity was determined by titration using 0.1 N NaOH to final pH 8.5. TTA was expressed as ml 0.1 N NaOH per 10 g of sourdough. All measurements were performed in triplicate.

2.3. Enumeration of lactic acid bacteria

The number of colony forming lactic acid bacteria in sourdough samples was determined by plate counting. Five grams of sourdough and 45 ml of 0.85 % NaCl solution were added to a sterile 50 ml centrifuge tube and mixed on a Vortex for 5 minutes. Series of decimal dilutions were plated on MRS agar (LabM, UK) in triplicate. Plates were incubated at 30^{o}C in both aerobic and anaerobic conditions for 48 hours. The BD GasPak EZ System (Becton Dickinson Microbiology Systems, USA) was used to create anaerobic environment.

2.4. DNA extraction

The Whatman Indicating FTA MiniCards (GE Healthcare Ltd., UK) were used for DNA extraction from isolated colonies as described by manufacturer. DNA extracted with this method was used for Rep-PCR analysis.

A modified phenol-chloroform method described by Van der Meulen et al. (2007) and Camu et al. (2007) was used to extract DNA directly from sourdough samples and pure LAB

cultures for DGGE analysis and 16S rRNA gene sequencing. The bacterial pellet obtained from 5 g of sourdough or 1.5 ml of overnight culture was washed in 1 ml of TES buffer (6.7 % sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA) and resuspended in 300 µl STET buffer (8 % sucrose, 5 % Triton X-100, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA). 75 µl of TES lysis buffer containing 1170 U/ml mutanolysin and 100 mg/ml lysozyme and 100 µl of proteinase K (2.5 mg/ml) (all enzymes from Sigma-Aldrich Co. LLC., USA) were added and samples were incubated at 37°C for 1 hour. After incubation 40 µl of preheated (37 °C) 20 % SDS in TE buffer and a pinch of glass beads (diameter 150 to 212 µm) were added. Samples were mixed on vortex for 1 minute and incubated at 37°C for 10 minutes, followed by incubation at 65°C for 10 minutes. Two phenol-chloroform-isoamylalcohol (50:49:1) extractions and one chloroform-isoamylalcohol (49:1) extraction were carried out. DNA was precipitated from the aqueous phase by adding 0.1 volumes of 3 M sodium acetate and 2 volumes of cold 96 % ethanol and washed with 70 % ethanol. The pellet was dried at room temperature and resuspended in 50 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0, AppliChem GmbH, Germany).

2.5. Repetitive Element Palindromic PCR (Rep-PCR)

Rep-PCR (repetitive element palindromic PCR) with primer $(GTG)_5$ was performed essentially as described by De Vuyst et al., 2002. For each sourdough sample 40 colonies were analyzed by Rep-PCR. 20 colonies were picked from one aerobically incubated plate and another 20 colonies from one anaerobically incubated plate. Colony picking was performed successively from one sector of the plate. PCR reaction was carried out in 25 µl volume using the following cycle: preliminary denaturation 6 minutes at 95°C; amplification in 30 cycles: denaturation 94°C for 1 minute, annealing 1 minute at 40°C, extension 8 minutes at 65°C and final extension at 65°C for 16 minutes. Share of each LAB fingerprint

type among aerobically and anaerobically selected isolates was calculated as ratio of similar fingerprints to the number of analyzed colonies.

2.6. 16 rRNA gene sequencing analysis

One to two representatives of each Rep-PCR fingerprint group were subjected to 16S rRNA gene analysis. 16S rRNA gene fragments were amplified using universal primers 27f-YM (Frank et al., 2008) and 16R1522 (Weisburg et al., 1991) followed by column purification of the amplified fragment with GeneJET PCR Purification Kit (Fermentas, Lithuania) and set up for sequencing PCR reaction using BigDye Terminator v3.1 Cycle Sequencing Kit as described by manufacturer (Applied Biosystems, USA). The partial 16S rRNA gene sequences obtained (approx. 700 bp) were compared with GenBank database using the BLAST algorithm (National Center for Biotechnology Information, USA).

2.7. Denaturing Gradient Gel Electrophoresis (DGGE) analysis

For DGGE analysis DNA was amplified using primers F357GC and 518R as described by Gafan and Spratt (2005). DNA was extracted directly from the sourdough samples, pure cultures of identified LAB isolates and 10 kGy irradiated rye flour. Sterile dark rye flour type R1370 (Tartu Grain Mill Ltd., Estonia) was obtained by γ-irradiation at 10 kGy using a dosimetric system GEX WinDose (Centennial, CO, USA). Polyacrylamide gel (8 % acrylamide-N,N'-methylenebisacrylamide; 37.5:1) with a gradient from 35 to 70 % of urea and formamide (100 % corresponding to 40 % v/v formamide and 7 M urea) was used. Electrophoresis was performed with the INGENYphorU system (Ingeny BV International, The Netherlands) at constant voltage 75 V and temperature 60^{o}C for 18 hours. The gels were stained with ethidium bromide and photographed with the ImageQuant 400 system (GE Healthcare, USA). Bands of interest were excised and DNA eluted by incubating in TE buffer

overnight at 4°C. Eluted DNA was reamplified using same primers without the GC-clamp. DNA fragments obtained were cloned using InsTAclone PCR Cloning Kit (Fermentas, Lithuania) combined with the TransformAid Bacterial Transformation Kit (Fermentas, Lithuania). Cloned DNA fragments were sequenced using the M13 primer.

2.8. Determination of carbohydrate fermentation profiles and proteolytic activity of L. helveticus

Metabolic profiles of *L. helveticus* strains were determined with API 50 CH testing and proteolytic activity was studied with the API ZYM test (Humble et al., 1977) as described by the manufacturer (bioMérieux, France). Skim milk agar was used to assess the ability of the bacteria to hydrolyze casein (Marcy and Pruett, 2001). The test was performed at both 30 and 37°C in aerobic and anaerobic conditions. *L. helveticus* CH-1 (Chr. Hansen, Denmark) was used as a reference strain.

3. Results

3.1. Characterization of lyophilized starter and industrial sourdough

The number of colony forming LAB in lyophilized starter was 2×10^6 and 10^7 CFU g^{-1} in aerobic and anaerobic conditions, respectively. The counts were approximately two magnitudes lower compared to the freshly fermented sourdough (Table 1.). This may indicate that freeze-drying and storage during six years had a negative effect on the cultivability of LAB.

The industrial sourdough cycle was started by inoculation with the freeze-dried sourdough at final LAB concentration 10^6 CFU g^{-1} dough (Table 1). The number of LAB in the sourdough increased to 10^9 CFU g^{-1} already 24 h after inoculation. During the following seven months

the number of LAB stayed around 10^9 CFU g^{-1} (Table 1). Colony forming numbers were generally ten times lower on plates incubated in aerobic conditions (Table 1).

Acidity of the renewed sourdough quickly increased to 20 ml 0.1 N NaOH/10 g sourdough during the first 24 hours of incubation (Table 1). The acidity slightly decreased after third to fifth back-slopping step, presumably due to decreased fermentation time. After five weeks of back-slopping the pH and TTA values of the industrial sourdough were around 3.5 - 3.7 and 19 - 22, respectively (Table 1).

3.2. Microbial composition of the lyophilized starter and industrial sourdough samples

The microbial composition of sourdough samples was evaluated using Rep-PCR fingerprinting with $(GTG)_5$ primer. Altogether 40 LAB isolates were analyzed from each sample, 20 of which were picked from aerobically and 20 from anaerobically incubated plates. Three to six fingerprint types per sample were detected among LAB isolated in anaerobic conditions. LAB isolated in aerobic conditions were represented by one to three fingerprint types per sample. Fingerprint types of the aerobic isolates were not unique and coincided with those of anaerobic isolates (data not shown).

One or more representatives from each fingerprint group (Fig. 1) were subjected to partial sequencing of the 16S rRNA gene (Table S1). Bacteria belonging to *L. helveticus*, *L. panis*, *L. pontis* and *L. vaginalis* species were identified among isolates obtained from the lyophilized starter. Most of the randomly picked isolates belonged to species *L. helveticus* irrespectively from incubation conditions (Table 2). Species identified from the lyophilized starter were represented by one (*L. helveticus* N92 and *L. vaginalis* N1113) or two (*L. panis* N915, N1311 and *L. pontis* N139, N131) fingerprint types (Fig. 1, Table S2).

Representatives of *L. helveticus* species were the dominating aerobic isolates obtained from industrial rye sourdough samples during the seven months long observation period. They formed 17/20 to 20/20 of the isolates in aerobic conditions (Table 2). The subdominant population varied among the samples and consisted of LAB belonging to species *L. vaginalis*, *L. fermentum* or *L. paralimentarius*.

L. helveticus was also the dominating colony forming species among LAB isolated in anaerobic conditions. However, its proportion among colony forming bacteria gradually decreased as it was replaced by bacteria belonging to species *L. pontis* and *L. panis* (Table 2). Representatives of *L. vaginalis* and *L. casei* were sporadically detected among the subdominant population.

Three fingerprint patterns were detected for *L. helveticus* species (N720, N92 and E96), two fingerprint patterns for *L. panis (*N915, N1311), *L. pontis (*N139, N131) and *L. vaginalis* (N1113, N116) species and one fingerprint pattern for *L. casei* (N726), *L. fermentum* (E112) and L. *paralimentarius* (E712) species (Fig. 1). *L. helveticus* bacteria with N92 and E96 Rep-PCR profiles were isolated in both aerobic and anaerobic conditions, *L. helveticus* N720 fingerprint type was from a single isolate from sourdough sample obtained five weeks after the start of the new sourdough cycle. Only *L. helveticus* N92 bacteria were isolated from the lyophilized starter and they were also the dominating type among all *L. helveticus* isolates in the industrial sourdough during seven month of propagation (Table S2).

3.3. PCR-DGGE fingerprinting of LAB in the lyophilized starter and industrial sourdough samples

The culture independent PCR-DGGE analysis was used in parallel to plating and Rep-PCR fingerprinting to characterize changes in the microbial consortium of sourdough. Preliminary

PCR-DGGE analysis of the isolated LAB revealed that bacteria identified as *L. helveticus*, *L. vaginalis* and *L. fermentum* species produced a single separately migrating band on the gel. Bacteria belonging to *L. panis, L. pontis* and *L. casei* species, however, showed multiple bands, possibly due to the heterogeneity of 16S rRNA gene operons (Fig. 2). The presence of one band in the control sample (10 kGy irradiated flour) indicated that plant genome can be amplified with the universal primers F357-GC and 518R. Sequencing analysis of this band showed similarity to mitochondrial cereal DNA.

PCR-DGGE fingerprinting of the freeze-dried sourdough starter and industrial sourdough samples revealed a remarkable similarity in band patterns during the whole seven month period. Bands with positions corresponding to the dominating LAB species *L. helveticus*, *L. pontis* and *L. panis* were observed in all fingerprinting patterns. The PCR-DGGE band patterns also showed no major changes in the microbial population of the industrial sourdough during the seven months of propagation. Only the detectable appearance of *L. vaginalis* and decrease of *L. panis* as a result of daily propagation could be suggested based on DGGE analysis of the industrial sourdough (Fig. 2). Identity of all bands was additionally confirmed by sequencing analysis.

3.4. Metabolic profiles of Lactobacillus helveticus isolates

16S rRNA gene sequencing and PCR-DGGE analysis determined *L. helveticus* as dominating species in the lyophilized starter and industrial sourdough. However, *L. helveticus* is used as a starter in the dairy industry and is not generally known as a sourdough LAB. API 50 CH test was used to evaluate metabolic potential of the *L. helveticus* isolates in parallel to *L. helveticus* CH-1 strain used in the dairy industry.

Three *L. helveticus* strains (*L. helveticus* N720, *L. helveticus* N92 and *L. helveticus* E96) representing different fingerprint groups were studied. All three strains isolated in this study could ferment glucose, esculin ferric citrate, saccharose, salicin and cellobiose (Table 3). Maltose and mannose were fermented by *L. helveticus* E96 and N720, but not by the N92 strain representing dominating fingerprinting group of the sourdough. The *L. helveticus* N720 also fermented galactose and N-acetyl glucosamine. None of the three *L. helveticus* isolated from the rye sourdough could ferment lactose, contrary to *L. helveticus* CH-1 strain, which fermented lactose, mannose, fructose, glucose and galactose.

We determined the proteolytic activity of *L. helveticus* N92, E96 and N720 strains and the reference dairy strain *L. helveticus* CH-1 using skim milk agar test and API ZYM test panel. Skim milk agar test showed that only the reference strain CH-1 was able to hydrolyze casein (Table 4). None of the strains had trypsin or α-chymotrypsin activity. The activity of specific arylamidases that catalyze the hydrolysis of N-terminal amino acid from peptides, amides or arylamides differed among the tested strains (Table 4).

4. Discussion

L. helveticus was the dominant LAB species in the lyophilized starter and industrial sourdough samples. Unlike *L. panis* (Wiese et al., 1996) and *L. pontis* (Vogel et al., 1994) also detected among dominant LAB population of the studied sourdough, *L. helveticus* is not a common sourdough LAB. It is often found in fermented milk products and is used as a cheese starter culture (Broadbent et al., 2011; Slattery et al. 2010). So far *L. helveticus* has been isolated from Sudanese sorghum sourdough (Hamad et al., 1997), traditional wheat or wheat-rye sourdoughs of East-Flanders region in Belgium (Scheirlinck et al., 2008) and some commercial starters (Moroni et al., 2010). The metabolic profiles of *L. helveticus* isolated

from sourdough in our work significantly differed from those determined for type strain and the dairy starter.

BLAST analysis of partial 16S rRNA gene sequences of isolates N92, E96 and N720 (GenBank ID: HM641233, HM623785 and HM641232, respectively) first determined *L. suntoryeus* as the closest match (data not shown). *L. suntoryeus* is a recently identified species (Cachat and Priest, 2005), which afterwards was claimed to be a later synonym of *L. helveticus* (Naser et al., 2006). Representatives of *L. suntoryeus* species were first isolated from late stages of barley fermentation in Japanese and Scottish malt whisky distilleries (Cachat and Priest, 2005) and similarly to *L. helveticus* belong to thermophilic homofermentative lactobacilli (Cachat and Priest, 2005).

In spite of the high similarity of *L. helveticus* and *L. suntoryeus* at sequence level, which determined them as one species, they formed distinguishable groups based on SDS-PAGE protein profiles and metabolic profiling (Naser et al., 2006). *L. helveticus* type strains are able to ferment lactose and/or galactose (Sharpe, 1981; Naser et al., 2006; API test identification table; this work). In contrast, none of the strains isolated from whisky distilleries (and primarily identified as *L. suntoryeus*) could ferment lactose (Naser et al., 2006). Instead they were able to ferment disaccharides cellobiose and sucrose, compounds found in plant material, and salicin, which is a β-glucoside produced from bark. Similarly, *L. helveticus* strains isolated in our work were able to utilize cellobiose, sucrose and salicin, but not lactose or galactose. The only exception was *L. helveticus* N720, which additionally utilized galactose, thereby resembling another metabolically versatile *L. helveticus* strain R0052 (Naser et al., 2006).

L. helveticus are known to have a vast proteolytic system containing several proteinases that are important for the technological potential of these bacteria in the dairy industry

(Christensen et al., 1999). Rapid growth in milk relies on complex proteolytic system, whose collective function involves the release of essential aminoacids from large proteins such as casein (Christensen et al., 1999). However, none of the *L. helveticus* strains we isolated from sourdough were able to hydrolyze casein.

Assumingly genomic rearrangements have occurred in the adaptation process of *L. helveticus* to specific environments. *L. helveticus* genome encompasses more than 200 insertion sequence (IS) elements (Callanan et al., 2008) which are responsible for the heterogeneity among *L. helveticus* strains (Kaleta et al., 2010) and have been associated with truncations in genes associated with cellobiose transport (Callanan et al., 2007) and the *lac* gene cluster (Callanan et al., 2005). Sequencing of *L. helveticus* N92, N720 and E96 genomes is in progress to resolve the adaptation mechanisms to the sourdough environment.

L. helveticus remained dominating in the microbial population of industrial sourdough during seven months of daily back-slopping. However, its proportion among cultivable population decreased over time. Unlike the culture dependent method, DGGE analysis did not reveal significant changes in the microbial composition of industrial sourdough or in the species ratio. All three dominant species (*L. helveticus*, *L. panis* and *L. pontis*) and also *L. vaginalis* were detected with DGGE analysis. Representatives of *L. vaginalis* were also detected in the lyophilized starter used for renewing the sourdough cycle.

Culture independent methods, like DGGE, have several advantages compared to culture dependent methods in the study of sourdough, since LAB from continuously cycled sourdough can have low colony forming ability (De Vuyst et al., 2002, Iacumin et al., 2009). Also, media used for isolation of sourdough lactobacilli quantitatively and qualitatively influences the microbial population that is detected (Vera et al., 2009).

Although same LAB fingerprint types were isolated from aerobically and anaerobically incubated agar plates, the colony numbers were generally ten times higher on anaerobically grown plates. Additional experiments revealed that most of the isolated LAB were sensitive to oxygen (Mihhalevski et al., 2011). The industrial sourdough studied is characterized by high water content and large volumes (up to 800 kg), which limit the specific transport rate of oxygen from dough surface. Reversible sorption of oxygen by dough starch and gluten leads to low levels of dissolved oxygen in flour water slurry (Xu, 2001). Thus the microbial consortium has adapted to semi-anaerobic conditions.

Development of the microbial community in sourdough greatly depends on cycling conditions: fermentation temperature, type of flour, back-slopping rate and frequency (Hammes et al., 1996; Vogel et al., 1996; Hammes and Gänzle, 1998). Commercial starters may not be sufficiently adapted to cycling conditions and are consequently outcompeted by indigenous bacteria entering the sourdough cycle from the environment. However, if the strains are obtained from a cycle with similar parameters, like in this study, the consortium can be stable even after several months of back-slopping. Thus, using an established and adapted microbial consortium as a starter could be a good option for bakeries.

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References

Broadbent, J. R., Cai, H., Larsen, R. L., Hughes, J. E., Welker, D. L., De Carvalho, V. G., Tompkins, T. A., Ardö, Y., Vogensen, F., De Lorentiis, A., Gatti, M., Neviani, E. and Steele, J. L. (2011) Genetic diversity in proteolytic enzymes and amino acid metabolism among *Lactobacillus helveticus* strains. *Journal of Dairy Science* 94 (9), 4313-28.

Cachat, E. and Priest, F. G. (2005) *Lactobacillus suntoryeus* sp. nov., isolated from malt whiskey distilleries. *International Journal of Systematic and Evolutionary Microbiology* 55 (1), 31-34.

Callanan, M. J. Beresford, T. P. and Ross, R. P. (2005) Genetic diversity in the lactose operons of *Lactobacillus helveticus* strains and its relationship to the role of these strains as commercial starter cultures. *Applied Environmental Microbiology* 71 (3), 1655-1658.

Callanan, M. J., Ross, R. P. and Beresford, T. P. (2007) Insertion sequence elements as mediators of strain diversity in *Lactobacillus helveticus*. *International Journal of Food Microbiology* 120, 120-123.

Callanan, M., Kaleta, P., O'Callaghan, J., O'Sullivan, O., Jordan K., McAuliffe, O., Sangrador-Vegas, A., Slattery, L., Fitzgerald, G. F., Beresford, T. and Ross, R. P. (2008) Genome sequence of *Lactobacillus helveticus*, an organism distinguished by selective gene loss and insertion sequence element expansion. *Journal of Bacteriology* 190 (2), 727-35.

Camu, N., De Winter, T., Verbrugghe, K., Cleenwerck, I., Vandamme, P., Takarama, J. S., Vancanneyt, M. and De Vuyst, L. (2007) Dynamics and biodiversity of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. *Applied and Environmental Microbiology* 73 (6), 1809-1824.

Christensen, J. E., Dudley, E. G., Pederson, J. R. and Steele, J. L. (1999) Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* 76, 217–246.

Corsetti, A. and Settanni, L. (2007) Lactobacilli in sourdough fermentation. *Food Research International* 40, 539-558.

De Vuyst, L., Schrijvers, V., Paramithiotis, S., Hoste, B., Vancanneyt, M., Swings, J., Kalantzopoulos, G., Tsakalidou, E. and Messens, W. (2002) The biodiversity of lactic acid bacteria in Greek traditional wheat sourdoughs is reflected in both composition and metabolite formation. *Applied Environmental Microbiology* 68, 6059-6069.

De Vuyst, L. and Neysens, P. (2005). The sourdough microflora: biodiversity and metabolic interactions. *Trends Food Science and Technology* 16, 43-56.

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A. and Olsen, G. J. (2008) Critical Evaluation of Two Primers Commonly Used for Amplification of Bacterial 16S rRNA Genes. *Applied and Environmental Microbiology* 74 (8), 2461-2470.

Gafan, G. P. and Spratt, D. A. (2005) Denaturing gradient gel electrophoresis gel expansion (DGGEGE) - an attempt to resolve the limitations of co-migration in the DGGE of complex polymicrobial communities. *FEMS Microbiology Letters* 253 (2), 303-307.

Hamad, S. H., Dieng, M., C., Ehrmann, M. A. and Vogel, R. F. (1997) Characterization of the bacterial flora of Sudanese sorghum flour and sorghum sourdough. *Journal of Applied Microbiology* 83 (6), 764-770.

Hammes, W. P., Stolz, P. and Gänzle, M. (1996) Metabolism of lactobacilli in traditional sourdoughs. *Advances in Food Science* 18, 176-184.

Hammes, W. P. and Gänzle, M. G. (1998) Sourdough breads and related products. In Microbiology of Fermented Foods, vol. 1. ed. Woods, B. J. B. pp. 199-216. London: Blackie Academic.

Hansen, A. S. (2004) Sourdough bread. In Handbook of Food and Beverage Fermentation Technology ed. Hui, Y. H., Meunier-Goddik, L., Hansen, A. S., Josephsen, J., Nip, W.-K., Stanfield, P. S. and Toldra, F. pp. 840-870. New York: Marcel Dekker, Inc.

Humble, M. W., King, A. and Phillips, I. (1977) API ZYM: a simple rapid system for the detection of bacterial enzymes. *Journal of Clinical Pathology* 30, 275-277.

Iacumin, L., Cecchini, F., Manzano, M., Osualdini, M., Boscolo, D., Orlic, S. and Comi, G. (2009) Description of the microflora of sourdoughs by culture-dependent and culture-independent methods. *Food Microbiology* 26, 128-135.

Kaleta, P., O'Callaghan, J., Fitzgerald, G. F., Beresford, T. P. and Ross, R. P. (2010) Crucial role for insertion sequence elements in *Lactobacillus helveticus* evolution as revealed by interstrain genomic comparison. *Applied Environmental Microbiology* 76 (1), 212-220.

Marcy, J. A. and Pruett, W. P. (2001) Proteolytic microorganisms. In Compendium of Methods for the Microbiological Examination of Foods ed. Downes, F. P. and Ito, K. pp. 183-186. Washington: American Public Health Association.

Meroth, C. B., Walter, J., Hertel, C., Brandt, M. J. and Hammes, W. P. (2003) Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturation gradient gel electrophoresis. *Applied Environmental Microbiology* 69, 475-482.

Mihhalevski, A., Sarand, I., Viiard, E., Salumets, A. and Paalme, T. (2011) Growth characterization of individual rye sourdough bacteria by isothermal microcalorimetry. *Journal of Applied Microbiology* 110, 529-540.

Moroni, A. V., Arendt, E. K., Morrissey, J. P. and Dal Bello, F. (2010) Development of buckwheat and teff sourdoughs with the use of commercial starters. *International Journal of Food Microbiology* 142, 142-148.

Naser, S. M., Hagen, K. E., Vancanneyt, M., Cleenwerck, I., Swings, J. and Tompkins, T. A. (2006) *Lactobacillus sunteryeus* (Cachat and Priest, 2005) is a later synonym of *Lactobacillus helveticus* (Orla-Jensenj 1919) Bergey et al. 1925 (Approved lists 1980). *International Journal of Systematic and Evolutionary Microbiology* 56 (2), 355-360.

Rosenquist, H. and Hansen, A. S. (2000) The microbial stability of two bakery sourdoughs made from conventionally and organically grown rye. *Food Microbiology* 17, 241-250.

Salim-ur-Rehman, Paterson, A. and Piggott, J. R. (2006) Flavour in sourdough breads: a review. *Trends Food Science Technology* 17, 557-566.

Scheirlinck, I., Van der Meulen, R., Van Schoor, A., Vancanneyt, M., De Vuyst, L., Vandamme, P. and Huys, G. (2007) Influence of geographical origin and flour type on diversity of lactic acid bacteria in traditional Belgian sourdoughs. *Applied Environmental Microbiology* 73 (19), 6262-6269.

Scheirlinck, I., Van der Meulen, R., Van Schoor, A., Vancanneyt, M., De Vuyst, L., Vandamme, P. and Huys, G. (2008) Taxonomic structure and stability of the bacterial community in Belgian sourdough ecosystems as assessed by culture and population fingerprinting. *Applied Environmental Microbiology* 74 (8), 2414-2423.

Sharpe, M.E. (1981) The genus *Lactobacillus*. In The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria, vol. 2. ed. Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Schlegel, H.G. pp. 1653-1679. New York: Springer.

Siragusa, S., Di Cagno, R., Ercolini, D., Minervini, F., Gobbetti, M. and de Angelis, M. (2009) Taxonomic structure and monitoring of the dominant population of lactic acid bacteria during wheat flour sourdough type I propagation using *Lactobacillus sanfranciscensis* starters. *Applied Environmental Microbiology* 75, 1099-1109.

Slattery, L., O'Callaghan, J., Fitzgerald, G. F., Beresford, T. and Ross, R. P. (2010) Invited review: *Lactobacillus helveticus* - a thermophilic dairy starter related to gut bacteria. *Journal of Dairy Science* 93 (10), 4435-4454.

Van der Meulen, R., Scheirlinck, I., Van Schoor, A., Huys, G., Vancanneyt, M., Vandamme, P. and De Vuyst, L. (2007) Population dynamics and metabolite target analysis of lactic acid bacteria during laboratory fermentations of wheat and spelt sourdoughs. *Applied Environmental Microbiology* 73 (15), 4741-4750.

Vera, A., Rigobello, V. and Demarigny, Y. (2009) Comparative study of culture media used for sourdough lactobacilli. *Food Microbiology* 26, 728-733.

Vogel, R. F., Böcker, G., Stolz, P. and 7 other authors (1994) Identification of lactobacilli from sourdough and description of *Lactobacillus pontis* sp. nov. *International Journal of Systematic Bacteriology* 44, 223–229.

Vogel, R. F., Müller, M., Stolz, P. and Ehrmann, M. (1996) Ecology in sourdoughs produced by traditional and modern technologies. *Advances in Food Science* 18, 152-159.

Weckx, S., Van der Meulen, R., Maes, D., Scheirlinck, I., Huys, G., Vandamme, P. and De Vuyst, L. (2010) Lactic acid bacteria community dynamics and metabolite production of rye sourdough fermentations share characteristics of wheat and spelt sourdough fermentations. *Food Microbiology* 27, 1000-1008.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. (1991) Ribosomal DNA Amplification for Phylogenetic Study. *Journal of Bacteriology* 173, 697-703.

Wiese, B. G., Strohmar, W., Rainey, F. A. and Diekmann, H. (1996) *Lactobacillus panis* sp. nov., from sourdough with a long fermentation period. *International Journal of Systematic Bacteriology* 46, 449–453.

Xu, F. (2001) Adsorption of oxygen gas by hydrated wheat flour. *LWT - Food Science and Technology* 34 (2), 89-94.

Supporting Information

Table S1. Similarity values obtained with BLAST analysis for different fingerprint groups of isolates

Table S2. Frequency of different Rep-PCR fingerprinting groups in the analyzed samples

Table 1. pH, acidity and bacterial counts in samples of industrial sourdoughs after renewing. Standard deviations are shown with (±).

Number of back-slopping propagations	Time from the start of renewal	pH	TTA ml 0.1 N NaOH / 10 g sourdough	Aerobic LAB counts CFU g $^{-1}$	Anaerobic LAB counts CFU g $^{-1}$
0	0 hours	4.79 ± 0.11	4.98 ± 0.43	8.91 ± 0.23 ×	7.08 ± 0.07 ×
1	24 h	3.63 ± 0.06	20.74 ± 0.48	1.95 ± 0.02 ×	7.59 ± 0.04 ×
2	43 h	3.53 ± 0.14	21.27 ± 0.15	4.27 ± 0.00 ×	1.10 ± 0.01 ×
3	55 h	3.54 ± 0.14	17.66 ± 0.09	2.40 ± 0.03 ×	6.46 ± 0.08 ×
4	67 h	3.54 ± 0.12	17.34 ± 0.38	2.82 ± 0.03 ×	1.07 ± 0.01 ×
5	77 h	3.66 ± 0.13	15.71 ± 0.27	1.29 ± 0.00 ×	5.62 ± 0.07 ×
40*	2 weeks	ND	ND	1.17 ± 0.05 ×	ND
100*	5 weeks	3.67 ± 0.06	22.30 ± 0.56	4.57 ± 0.02 ×	6.61 ± 0.05 ×
250*	14 weeks	3.71 ± 0.01	18.89 ± 0.05	4.68 ± 0.06 ×	1.10 ± 0.01 ×

300*	16 weeks	3.63 ± 0.02	19.78 ± 0.07	1.29 ± 0.00 ×	1.41 ± 0.01 ×
350*	18 weeks	3.63 ± 0.04	21.30 ± 0.41	1.29 ± 0.00 ×	6.92 ± 0.05 ×
550*	34 weeks	3.53 ± 0.02	21.84 ± 0.30	3.31 ± 0.03 ×	1.20 ± 0.00 ×
600*	35 weeks	3.49 ± 0.02	21.28 ± 0.08	2.19 ± 0.02 ×	1.41 ± 0.02 ×

* - approximate numbers

ND – not determined

Table 2. Frequency of different species isolated from the lyophilized sourdough starter and the industrial sourdough samples from MRS plates incubated in aerobic (+) and anaerobic (-) environment. Share of each LAB is given as number of identified isolates per 20 analyzed colonies.

LAB species	Lyophilized		77 hours		5 weeks		14 weeks		18 weeks		34 weeks		35 weeks	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-
L. helveticus	19	15	18	2	18	17	20	13	17	9	20	3	20	3
L. panis	-	3	-	2	-	-	-	7	-	4	-	6	-	2
L. pontis	-	2	-	-	-	-	-	-	-	4	-	11	-	15
L. vaginalis	1	-	-	-	-	-	-	-	2	3	-	-	-	-
L. reuteri	-	-	-	14	-	-	-	-	-	-	-	-	-	-
L. casei/paracasei	-	-	-	1	-	3	-	-	-	-	-	-	-	-
L. fermentum	-	-	2	-	-	-	-	-	1	-	-	-	-	-
L. paralimentarius	-	-	-	-	2	-	-	-	-	-	-	-	-	-

Table 3. Metabolic profiles of *L. helveticus* strains based on API 50CH.

Carbohydrate in API 50CH	*L. helveticus N720*	*L. helveticus N92*	*L. helveticus E96*	*L. helveticus CH-1*	*L. helveticus profile* API †
D-galactose	+	-	-	+	+
D-glucose	+	+	+	+	+
D-fructose	+	-	-	+	+
D-mannose	+	-	+	+	+
D-lactose	-	-	-	+	+
Esculin ferric citrate	+	+	+	-	-
N-acetyl glucoseamine	+	-	-	-	+
D-saccharose	+	+	+	-	-
D-cellobiose	+	+	+	-	-
D-maltose	+	-	+	-	+
Salicin	+	+	+*	-	-

* positive after 72 hours incubation

† metabolic profile of *L. helveticus* in API test identification table

Table 4. Proteolytic activity of *L. helveticus isolates*. Weak reaction is indicated with 'v'.

Enzyme tested	*L. helveticus* N720	*L. helveticus* N92	*L. helveticus* E96	*L. helveticus* CH-1
Leucine arylamidase	+	+	+	+
Valine arylamidase	-	+	+	-
Cystine arylamidase	v	-	-	+
Trypsin	-	-	-	-
α-chymotrypsin	-	-	-	-
Casease *	-	-	-	+

* Activity of enzymes was determined by API ZYM method, except for casease, which was detected by skim milk agar testing.

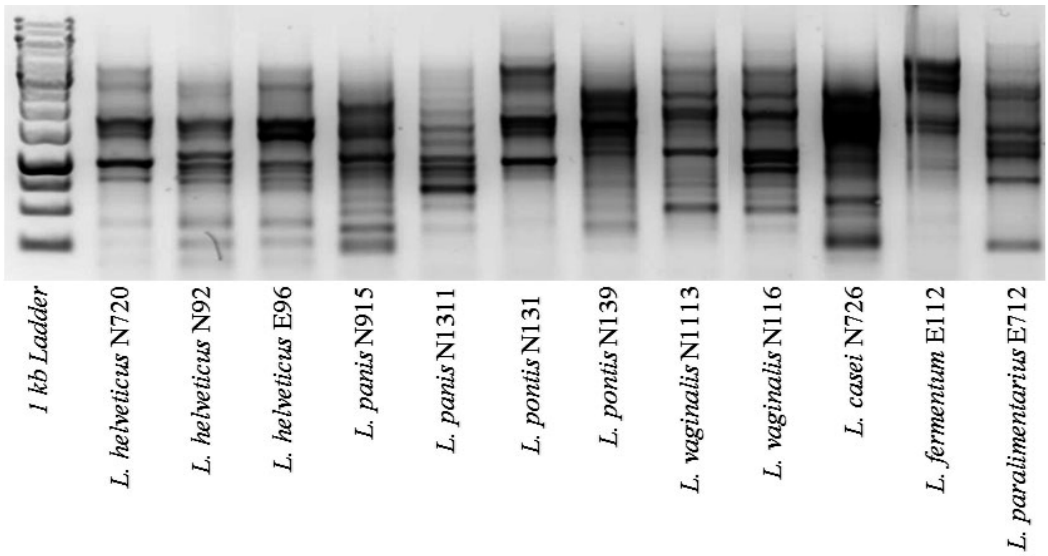

Figure 1. Rep-PCR profile of fingerprint types isolated from the industrial sourdough samples. 1 kb DNA Ladder is used as reference in the first lane.

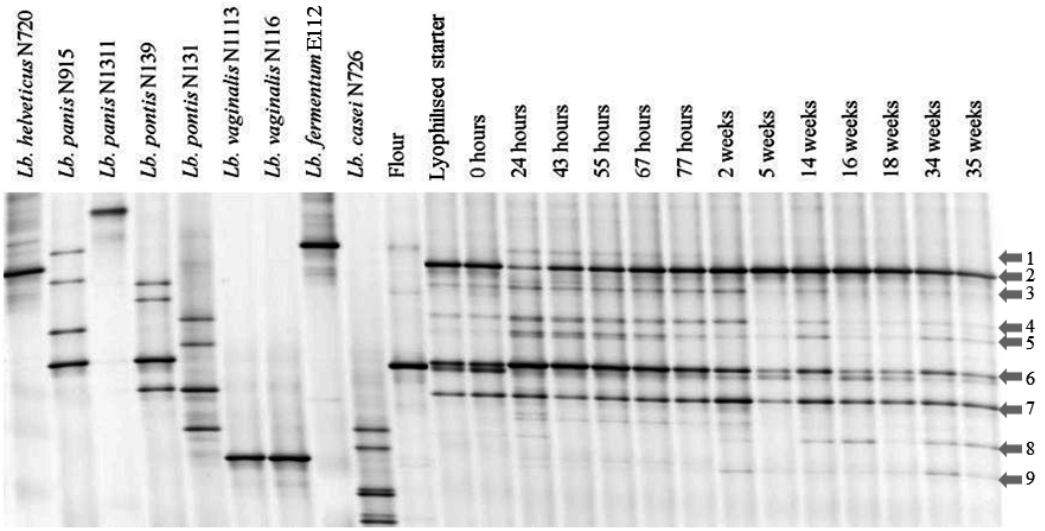

Figure 2. Dynamics of the microbial population in the industrial rye sourdough during seven months of propagation. Time from the start of the renewal is shown on top. Position of strain-specific and cereal-specific DGGE bands are shown in the left part of the figure. Numbers corresponding to bands identified by 16S rRNA gene sequencing: 1 – *L. panis*, 2 – *L. helveticus*, 3 – *L. panis*, 4 – *L. pontis*, 5 – *L. panis*, 6 – *L. panis*, 7 – *L. pontis*, 8 – *L. pontis* and 9 – *L. vaginalis*.

PUBLICATION IV

Mihhalevski A, Nisamedtinov I, Hälvin K, Ošeka A, Paalme T

Stability of B-complex vitamins and dietary fiber during rye sourdough bread production

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Stability of B-complex vitamins and dietary fiber during rye sourdough bread production

Anna Mihhalevski [a,b,*], Ildar Nisamedtinov [a,b], Kristel Hälvin [a,b], Aleksandra Ošeka [a,b], Toomas Paalme [a,b]

[a] Competence Center of Food and Fermentation Technologies, Akadeemia tee 15b, 12618 Tallinn, Estonia
[b] Department of Food Processing, Tallinn University of Technology, Ehitajate tee 5, 19086 Tallinn, Estonia

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ABSTRACT

The stability of vitamers: thiamine, riboflavin, nicotinic acid, nicotinamide, pantothenic acid, pyridoxine and pyridoxal, as well as soluble and insoluble dietary fiber was studied in a rye sourdough bread process. The vitamer concentrations were measured in raw materials (rye flours, white and red rye malt, yeast) and the rye sourdough breads made from them by means of LC–MS and stable isotope dilution assay. The content of dietary fiber was determined using a standard enzymatic-gravimetric method. During baking, the concentration of vitamins decreased by 20–45% in the case of thiamine, 25–50% in the case of nicotinic acid, 45–65% in the case of pyridoxal in both breads, 50% in the case of riboflavin and 15% in the case of pyridoxine only in fine rye bread. In contrast, the content of nicotinamide increased during processing by ten fold, presumably due to microbial activity during sourdough fermentation. The ratio of soluble to insoluble dietary fiber increased during rye sourdough processing.

1. Introduction

Rye sourdough bread is a traditional and popular bread in North–West Russia, the Baltic states, Finland, Denmark, and the North of Germany. Rye sourdough bread is industrially produced by mixing sourdough with flour, followed by fermentation with yeast (leavening), and baking. Rye bread is a recommended part of the diet because it is a good source of various bioactive substances, including B-complex vitamins and dietary fiber.

In industrial product labeling practice, the calculation of the content and composition of vitamins and fiber is based on the composition of their ingredients. However, this may result in incorrect estimations, mostly due to variations in the vitamins and fiber content of the particular ingredients used, but also due to chemical changes that occur during processing.

Dietary fiber is an indigestible complex of carbohydrates and lignin. It is an important functional component, the content of which can change during rye bread processing. Insoluble dietary fiber compounds in rye include cellulose (1–3 %), lignin (1–2 %) (Liukkonen et al., 2006) and the insoluble part of arabinoxylans. Rye grain contains 8–12 % of arabinoxylans, fructan (4.5–6.4%) and β-glucans (1–3%) (Hansen et al., 2003; Karppinen et al., 2003; Liukkonen et al., 2006). The soluble fibers are fructo-oligosacchrides 1-kestose (0.6%), 1,1-kestotetraose (0.3%), and 1,1,1-kestopentaose (0.3%) (Karppinen et al., 2003). Amount of water-extratable β-glucans is 1.2% in rye (Aura et al., 2007).

Enzymatic as well as thermal treatment can modify the ratio between soluble and insoluble fibers during bread making (Elleuch et al., 2011). During flour hydration, several intrinsic hydrolytic enzymes of cereals (α-amylase, β-xylosidase, α-L-arabinofuranosidase, cinnamoyl esterases, D-xylosidase, β-glucanases and endo-D-xylanase) are activated (Boskov Hansen et al., 2002). Also the enzymes with β-glycolytic activity from lactic acid bacteria (Axelsson, 2007) and yeast (Hauf et al., 2000) can affect the fiber concentration during rye bread processing. The most commonly used methods for the determination of dietary fiber in cereals are enzymatic-gravimetric methods (Lee et al., 1992).

The term *vitamin* refers to a number of *vitamer* compounds with the same biological activity as the respective vitamin (Table 1). Unfortunately, the complete list of vitamers with respective vitamin activity for mammals is still not clear. Several vitamins during food processing, storage and digestion are derived from cofactors as the result of enzymatic activities in both the food matrix and the digestive tract. For example, 2–10% of the vitamin B1 pool in cereals is present as its phosphorylated form thiamine diphosphate (TDP) (Buchholz et al., 2011), which is hydrolyzed by different phosphatases in the human gastrointestinal tract into

* Corresponding author. Competence Center of Food and Fermentation Technologies Akadeemis tee 15b, Tallinn, 12618, Estonia. Tel.: +372 6408201; fax: +372 6408282.
E-mail address: anna@tftak.eu (A. Mihhalevski).

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Table 1
Vitamins and corresponding vitamers.

Vitamin	Vitamers
B1, thiamine	Thiamine
	Thiamine monophosphate (TMP)
	Thiamine diphosphate (TDP)
	Thiamine triphosphate (TTP)
	2-(1-hydroxyethyl)thiamin (HET)
B2, riboflavin	Riboflavin
	Flavin mononucleotide (FMN)
	Flavin adenine dinucleotide (FAD)
B3, niacin	Nicotinic acid
	Nicotinamide
	Nicotinamide adenine dinucleotide (NAD)
	Nicotinamide adenine dinucleotide phosphate (NADP)
	Nicotinic acid adenine dinucleotide (NAAD)
	Nicotinic acid adenine dinucleotide phosphate (NAADP)
	N-ribosyl nicotinamide
	N-ribosyl nicotinic acid
B5, panthothenic acid	Pantothenic acid
	Coenzyme A (CoA)
	Phosphopantothenic acid
	Pantetheine
	Phosphopantetheine
B6, pyridoxine	Pyridoxine
	Pyridoxal
	Pyridoxamine
	Pyridoxine phosphate (PNP)
	Pyridoxal phosphate (PLP)
	Pyridoxamine phosphate (PMP)
	5′-O-β-D-glucopyranosyl pyridoxine

thiamine (Rindi and Laforenza, 2000). Similarly, the phosphorylated forms of riboflavin and vitamin B6 can also be found in nature (Cataldi et al., 2002; Roth-Maier et al., 2002).

In practice, the content of B-complex vitamins is expressed as the concentrations of their most simple commercially available chemical forms (e.g. B1 = thiamine chloride hydrochloride, B2 = riboflavin, niacin = nicotinic acid + nicotinamide, expressed as nicotinic acid, B5 = calcium pantothenate, B6 = pyridoxine + pyridoxal + pyridoxamine, expressed as pyridoxine hydrochloride) which often serve as a starting point for the synthesis of respective cofactor(s) in cells. Thus, in most of the food databases, the B-complex vitamin contents (μg per 100 g of food) are expressed as thiamine, riboflavin, niacin, pantothenic acid and pyridoxine hydrochloride equivalents.

Information regarding the stability of B-complex vitamers during the course of rye sourdough bread-making processes is scarce. Thiamine is relatively stable at pH 2.0–4.0, which is a common pH range of the sourdough. In slightly acidic solutions it is stable up to the boiling point. However, during baking, 20–50 % losses of thiamine have been reported (Martinez-Villaluenga et al., 2009). Similarly, riboflavin is relatively heat-stable (Batifoulier et al., 2005). It has been reported that whole wheat bread made with yeast results in 30% enrichment in riboflavin due to the contribution of endogenous yeast riboflavin, while sourdough lactic acid fermentation without yeast did not result in any increase in the bread's riboflavin content (Batifoulier et al., 2005).

Nicotinamide and nicotinic acid are relatively stable during baking (Ottaway, 2002). A small part of chemically bound nicotinic acid (i.e. NAD) can be hydrolyzed by gastric juice and is thus converted into bioavailable forms (Wall and Carpenter, 1988). Pantothenic acid has good stability during most food processing operations, including baking (Ottaway, 2002). Pyridoxine, pyridoxal and pyridoxamine are relatively heat-stable under acidic conditions and very heat-labile under alkaline conditions (Leklem, 2001). The baking of bread can induce losses of up to 17% for vitamin B6 (Ottaway, 2002).

The current study was carried out to analyze the stability of soluble and insoluble dietary fiber and B-complex vitamers during rye sourdough bread making. To determine vitamer concentrations we used a high throughput LC–MS and stable isotope dilution assay.

2. Materials and methods

2.1. Materials and reagents

Whole grain rye flour R1800, dark rye flour R1370, fine rye flour R705, wheat flour W700, inactive (red) rye malt, active (white) rye malt and steel cut rye grains were obtained from Tartu Grain Mill Ltd. (Tartu, Estonia). The same lots of flour were used for the production of all bread recipes. The commercial rye bread containing dark rye flour (55%), active rye malt (1%) and inactive rye malt (1%), yeast (0.9%), and fine rye bread containing fine rye flour (42%), wheat flour (10%), dark rye flour (7%), active rye malt (1%), and yeast (1%) were obtained from a local bakery.

Dark rye flour (R1370) used for incubation and fermentation experiments was sterilized by γ-irradiation at 10 kGy using a dosimetric system GEX WinDose (Centennial, CO, USA). Bacterial strain *Lactobacillus panis* N915 was isolated from the rye sourdough (Mihhalevski et al., 2011). Fresh baker's yeast was kindly provided by AS Salutaguse Pärmitehas (Kohila vald, Estonia).

Certified reference material BCR 121 (wholemeal flour) was obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). The certified values of B-complex vitamers with uncertainties (μg/100 g dry matter) for BCR 121 were as follows: 364 ± 31 for vitamin B1 (expressed as thiamine equivalents) and 377 ± 84 for B6 (expressed as pyridoxine equivalents).

Acid washed Celite was obtained from Megazyme International Ireland Ltd. (Bray, Ireland). Acetone and acetonitrile (both HPLC grade) were obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland, UK), MES (2-(N-morpholino) ethanesulfonic acid), ammonium formate (puriss.p.a., for HPLC), HCl (37% puriss.p.a.) and TRIS (tris(hydroxymethyl) aminomethane) were obtained from Sigma–Aldrich Inc (St.Louis, MO, USA), NaOH was obtained from Lach-Ner (Neratovice, Czech Republic) and Sigma–Aldrich. High purity water was produced by a Millipore water purification system (Millipore S.A.S., Malsheim, France).

Nicotinamide (99.9%), nicotinic acid (99%), thiamine chloride hydrochloride (99%), pyridoxine-HCl (99.9%), pyridoxal-HCl (99%), Ca-pantothenate (99%) and riboflavin (99%), used as external standards were obtained from Sigma–Aldrich. The stable isotope labeled internal standards nicotinamide – [D_4, 98%], nicotinic acid – [D_4, 98%], thiamine-Cl – [$^{13}C_3$, 99%], pyridoxine-HCl – [$^{13}C_4$, 99%], were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Ca-pantothenate – [$^{13}C_3$,^{15}N, 97.1%], riboflavin – [$^{13}C_4$,$^{15}N_2$, 97.9%] and pyridoxal HCl – [D_3, 99%] were from Isosciences, LLC (King of Prussia, PA, USA).

α-amylase from *Aspergillus oryzae* (crude, Cat. No. A6211), β-glucosidase from almonds (crude, Cat. No. G0395), and potato acid phosphatase (Cat. No. P1146) were obtained from Sigma–Aldrich.

Thiamine monophosphate chloride dehydrate (99%), thiamine pyrophosphate chloride (≥97%), riboflavin 5′-monophosphate sodium salt dehydrate (75.9%), flavin adenine dinucleotide disodium salt hydrate (97%), β-nicotinamide adenine dinucleotide hydrate (98%), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (99%) and coenzyme A hydrate (99%), were obtained from Sigma–Aldrich.

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2.2. Sample preparation

After slicing, the breads were frozen in liquid nitrogen, freeze-dried, ground in a laboratory Waring blender HGBTWTS3 (Waring, Torrington, CT, USA) (particle size from 0.10 to 0.25 mm), packed into plastic bags, and stored at −20 °C until further analysis. Flours, malts and cut rye grains were stored at −20 °C. Steel cut rye grains were ground before analysis (particle size from 0.10 to 0.25 mm).

To study the effect of fermentation on the content of B-complex vitamers the 10 kGy irradiated rye flour was mixed in a ratio of 1:1 with a suspension of *L. panis* N915 in 0.5% NaCl (5×10^6–5×10^7 cfu/mL) using a Stomacher 400 Circulator (Seward Ltd., Worthing, UK) for 15 min at 100 RPM and incubated at 30 °C for 24 h. Control sourdough was made without lactic acid bacteria. After sourdough fermentation, the samples were freeze-dried and ground until further analysis.

2.3. Analytical methods

All samples were analyzed at least in three replicates.

2.3.1. Dry matter

The dry matter content of each sample was measured using a Halogen Moisture Analyser HR83 (Mettler Toledo, USA) and the ash content was determined according to AOAC method (923.03). Samples were treated in a muffle furnace at 550 °C for 10 h.

2.3.2. Total, soluble and insoluble dietary fiber

Total, soluble and insoluble dietary fiber content was measured according to the enzymatic-gravimetric method using an assay kit from Megazyme International Ltd. (Bary, Ireland). Samples were subjected to sequential enzymatic digestion by heat-stable α-amylase, protease, and amyloglucosidase. Insoluble dietary fiber was filtered from the hydrolyzate, washed, dried and weighed. Soluble dietary fiber was precipitated from the filtrate with four volumes of 95% ethanol, filtered and dried. For determination of total dietary fiber, the samples after enzymatic treatment were treated with four volumes of ethanol to precipitate soluble fiber. The residue was then washed, filtered, dried and weighed. Total, soluble and insoluble dietary fiber contents were corrected, based on the protein and ash residues.

2.3.3. Determination of B-complex vitamer concentrations

LC–MS combined with a stable isotope dilution assay was used for the analysis of B-complex vitamer concentrations in the samples. Freeze dried flour and bread samples were weighed (approx 1.0 g) into a 10 mL volumetric flask, filled up with a 0.05 M ammonium formate buffer (pH 4.5), mixed thoroughly, transferred to a 50 mL centrifuge tube and incubated with frequent shaking at 37 °C for 18 h. After incubation, each sample extract was centrifuged at 14 000 RPM for 5 min at 22 °C by Micro CL21R centrifuge (Thermo Scientific, Osterode, Germany) to remove insoluble debris and filtered through a Millex-LG 13 mm Philic PTFE 0.2 μm filter (Millipore, Carrigtwohill, Ireland). 1000 μL aliquots of this filtrate were then transferred to LC-MS sample vials containing 20 μL of internal standard solution containing approximately 20 mg/L of each labeled vitamer.

Fresh baker's yeast samples (approx 0.5 g, DM 21%, in four replicates) were weighted into 2 mL eppendorf tubes, 0.5 mL of 0.05 M ammonium formate buffer (pH 4.5) was added, vortexed, and held in the water bath at 70 °C for 30 min using frequent shaking. After heating, approximately 1.0 g of glass beads (425–600 μm, acid washed, G8772, Sigma–Aldrich) were added to the sample and yeast cells were disrupted using a Genie cell disrupter (Scientific Industries Inc., New York, USA) for 30 min. The disrupted yeast samples were transferred quantitatively into a 10 mL volumetric flask, filled up with 0.05 M ammonium formate buffer (pH 4.5), mixed thoroughly, incubated, centrifuged, filtered and prepared for LC–MS analysis as described above.

All vitamer analyses were performed using an ACQUITY UPLC® system, equipped with an ACQUITY UPLC HSS C-18 1.8 μm (2.1 × 150 mm) column and coupled with LCT Premier™ XE ESI TOF MS System (Waters, Milford, MA, USA). Elution was carried out using two eluents (A: water + 0.1% formic acid and B: acetonitrile + 0.1% formic acid) applied in the following gradient mode: 0–3 min 100% A; 3–8.5 min 80% A and 20% B; 8.5–10 min: 5% A and 95% B; 10–15 min 100% A. A flow rate of 0.25 mL/min and a sample injection of 5 μL were used. Mass spectrometry was carried out in positive ionization mode $[M + H]^+$ using a capillary voltage of 2000 V, sample cone voltage of 30 V, desolvation temperature of 300 °C and source temperature of 120 °C. Full scan mass spectra were acquired from *m/z* 100 to 500. Data were collected and processed using Mass Lynx 4.0 software (Waters, Milford, MA, USA).

The *m/z* ratios of 265.11 and 268.12 for thiamine, 377.15 and 383.16 for riboflavin, 123.06 and 127.08 for nicotinamide, 124.04 and 128.06 for nicotinic acid, 220.12 and 224.13 for pantothenic acid, 168.07 and 171.09 for pyridoxal, 170.08 and 174.10 for pyridoxine were used for the determination of unlabeled and labeled forms of vitamins, respectively.

Internal standard stock solutions were prepared by dissolving separately about 1 mg of each isotope labeled vitamer in 5 mL 0.05 M ammonium formate (pH 4.5) buffer. Internal standard solution (~20 mg/L) was prepared by combining aliquots of the stock solutions. Concentration of each vitamin in the sample was calculated using 7-point calibration curves. Calibration curve standards were prepared by adding 1000 μL aliquots of external standards solution with varying concentrations (0.01–0.9 mg/L) into the LC–MS vial containing 20 μL of internal standard solution. The calibration curves were constructed by relating the varying concentrations of unlabeled external standards to their relative response factors as determined by the ratio of the peak intensity of the unlabeled external standards to that of the corresponding labeled internal standard. Responses for all the vitamins were linear over the working range with correlation coefficients ranging between 0.9995 and 0.9999. The vitamers were separated by LC and the intensities of respective lines of natural abundance and isotope labeled internal standards in the MS spectra were measured (Fig. 1).

2.3.4. The effect of enzymatic treatment and acid hydrolysis on liberation of simple vitamers

To study the effect of enzymatic treatment on the extraction and liberation of B-complex vitamers, 10 mL of a mixture of enzyme solution in 0.05 M ammonium formate (pH 4.5) containing α-amylase (4.50 mg/mL), β-glucosidase (1.60 mg/mL) and acid phosphatase (0.16 mg/mL) was added to 1 g of sample before incubation described in Section 2.3.3. All results were enzyme blank corrected.

The phosphatase, pyrophosphatase, purine nucleosidase, pantetheine hydrolase activities of the enzymes in used enzyme preparations were determined by preparing the 25 μM solutions of following vitamer precursors: thiamine mono- and diphosphate (TMP, TDP), flavin mononucleotide and flavin adenine dinucleotide (FMN, FAD), NAD^+ and reduced nicotinamide adenine dinucleotide (NADH) and Coenzyme A (CoA). Each solution was incubated with 25 mL of acid phosphatase (0.12 mg/mL), β-glucosidase (1.2 mg/mL) or α-amylase (5.0 mg/mL) during 18 h at 37 °C followed by determination of liberated thiamine, riboflavin, nicotinamide, and pantothenic acid by LC–MS.

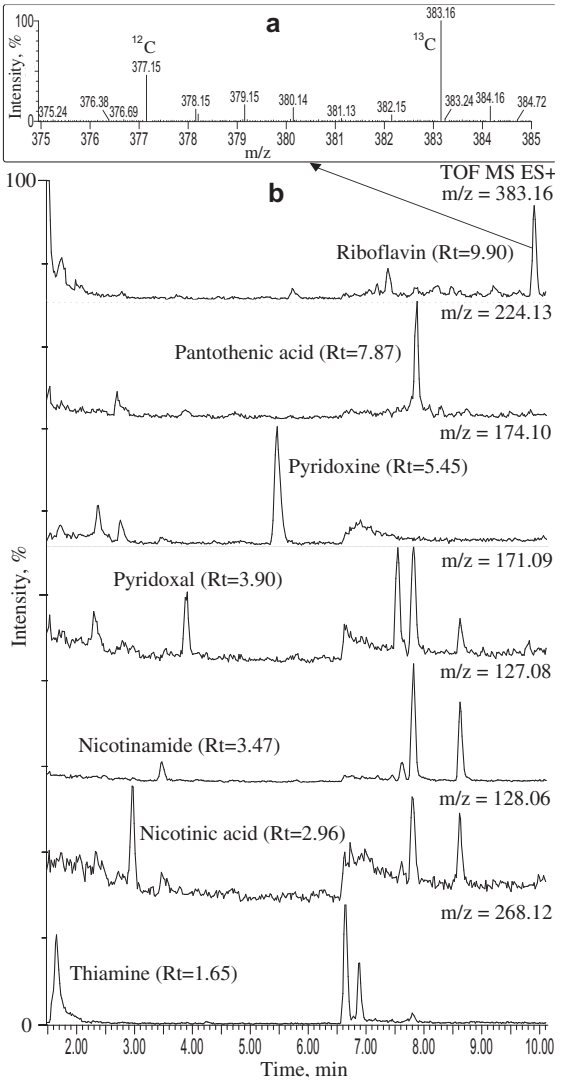

Fig. 1. TOF MS ES + spectrum of riboflavin in rye sourdough bread (a) and UPLC TOF MS ES + chromatograms of B-complex vitamers in rye sourdough bread (b).

Protease activity was determined using a protease assay kit (Calbiochem, USA): 100 μL of the single enzyme solution was combined with 50 μL of fluorescein thiocarbamoyl-casein (FTC-casein) and 50 μL of incubation buffer (200 mM Tris–HCl, pH 7.8, 20 mM $CaCl_2$, 0.1% NaN_3). Concentration of the enzyme solutions were as follows: α-amylase 0.225 mg/mL, β-glucosidase 1.2 mg/mL, acid phosphatase 0.12 mg/mL. Incubation was carried out at 37 °C for 24 h under subdued lighting conditions using continuous shaking. The reaction was stopped by the addition of 500 μL of 5% trichloroacetic acid. Denatured FTC-casein was separated from the reaction mixture by centrifugation (12 000 RPM for 5 min at room temperature) and the concentration of FTC-peptides in the supernatant was determined by absorbance at 492 nm. Protease activity was expressed relative to that of trypsin in BAEE (N-benzoyl-L-arginine ethyl ester) units.

The effect of acid hydrolysis on the liberation of vitamers from biologically active forms was studied as follows: 25 mL of TMP (25 μM), FMN (25 μM), TDP (25 μM), FAD (25 μM), NAD^+ (25 μM) or NADH (25 μM) in 0.1 M HCl were hydrolyzed in an autoclave at 121 °C for 30 min, followed by the determination of thiamine, riboflavin and nicotinamide.

2.4. Calculation of stability of nutrients during processing

The expected concentration of each nutrient in the bread was calculated on a dry mass basis by summing up the contributions from the individual ingredients. This involved measuring the amount of each nutrient found in each ingredient on a dry mass basis.

The recovery of B-complex vitamins after processing was calculated as the percentage of concentration measured in the bread sample divided by the value calculated according to the recipe.

3. Results

All results are presented on a dry matter (DM) basis.

3.1. Stability of dietary fiber during rye sourdough bread processing

The concentrations of soluble, insoluble and total dietary fiber were determined in all ingredients used for rye sourdough bread making as well as in the baked breads (Table 2). Similar values for total dietary fiber (15–17% DM) and for soluble fiber (2–3% DM) were found in whole grain rye flour, white rye malt, red rye malt and steel cut rye. In wheat flour and fine rye flour, the total dietary fiber was found to be only about 3% DM and 7% DM, respectively (Table 2). The soluble fiber content in fine rye flour (1.9% DM) was

Table 2
Total, soluble and insoluble dietary fibre in flours and breads.

Sample	Moisture %	Ash % DM	Total dietary fiber % DM	Soluble dietary fiber % DM	Insoluble dietary fiber % DM
Whole grain rye flour (R1800)	11.3 ± 0.2	1.4 ± 0.1	15.0 ± 0.5	2.34 ± 0.1	12.0 ± 0.8
Dark rye flour (R1370)	11.4 ± 0.2	1.1 ± 0.1	11.0 ± 0.6	2.3 ± 0.4	7.8 ± 0.8
Fine rye flour (R705)	11.8 ± 0.2	0.7 ± 0.03	6.6 ± 0.9	1.9 ± 0.2	3.6 ± 0.2
Wheat flour (W700)	12.6 ± 0.2	0.7 ± 0.04	2.7 ± 0.4	0.7 ± 0.2	1.2 ± 0.3
Non fermented rye malt	6.7 ± 0.2	1.5 ± 0.02	15.2 ± 0.8	2.2 ± 0.2	11.9 ± 1.3
Fermented rye malt	3.5 ± 0.2	1.6 ± 0.03	17.0 ± 1.8	3.1 ± 0.5	11.4 ± 2.0
Cut rye	11.1 ± 0.2	1.5 ± 0.1	16.3 ± 1.3	NT	12.8 ± 1.7
Rye bread					
Experimentally measured	42.4 ± 0.5	2.7 ± 0.01	11.1 ± 1.1	3.4 ± 0.2*	6.2 ± 0.3
Calculated			9.73 ± 0.5	2.02 ± 0.3	6.91 ± 0.7
Fine rye bread					
Experimentally measured	41.8 ± 0.2	2.3 ± 0.03	10.0 ± 0.7*	2.6 ± 0.4*	6.0 ± 0.5*
Calculated			6.02 ± 0.2	1.54 ± 0.1	3.42 ± 0.2

NT – not tested, * - experimental value is different from calculated value ($p < 0.05$).

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relatively high compared to that of wheat flour (0.7% DM). The highest concentration of soluble fiber in the raw materials studied was observed in fermented rye malt (3.1% DM).

The concentration of soluble fiber in the rye bread (2.0% DM) and in fine rye bread (1.5% DM), calculated on the basis of their recipes and on the fiber content in flours used were lower than the measured values (3.4 and 2.6% DM, respectively). This also applies to the experimentally determined total dietary fiber concentration of fine rye bread.

3.2. B-complex vitamers in cereal materials and rye bread

The concentrations of seven vitamers of B-complex vitamins: thiamine, riboflavin, nicotinic acid, nicotinamide, pantothenic acid, pyridoxine and pyridoxal were analyzed in reference wholemeal flour, different breads, as well as the raw materials used to make them using extraction with ammonium formate buffer and an LC–MS isotope dilution assay method. These vitamers were well resolved in the reversed phase column under the chromatographic conditions specified in Methods (Fig. 1). The MS spectrum of riboflavin in the internal standard is given as an example.

The concentration of B-complex vitamers in the reference material, raw materials, and breads prepared from them is given in Table 3.

The concentration of thiamine in the reference wholemeal flour, determined without enzymatic treatment, was 376 ± 39 μg/100 g DM (Table 3), which corresponds to the certified value (364 ± 31 μg/100 g DM) for vitamin B1, expressed as thiamine. Using enzymatic extraction the concentration of thiamine increased up to 394 ± 31 μg/100 g DM, which is not significantly higher ($p > 0.05$) than the reference value. On the contrary, the sum of pyridoxal and pyridoxine concentration (expressed as pyridoxine) was 226 ± 15 μg/100 g DM, which was significantly lower than that of the reference value 377 ± 84 μg/100 g DM. Enzymatic

Table 3
Content of B-complex vitamers in reference material, flours, sterile sourdoughs, and breads obtained using the extraction with and without enzyme mix. The recipe based values of vitamers in breads (calculated).

Samples	Thiamine	Riboflavin	Nicotinic acid	Nicotinamide	Pantothenic acid	Pyridoxal	Pyridoxine
	μg/100 g of DM						
Reference material BCR 121 (wholemeal flour)							
Certified value	364 ± 31	–	–	–	–	–	–
Free vitamins	376 ± 39^{a}	129 ± 25^{a}	615 ± 45^{a}	32 ± 1^{a}	765 ± 54^{a}	30 ± 2^{a}	196 ± 13^{a}
Enzymatic extraction	394 ± 31^{a}	325 ± 135^{b}	391 ± 28^{b}	58 ± 2^{b}	658 ± 47^{a}	28 ± 4^{a}	189 ± 16^{a}
Steel cut rye							
Free vitamins	230 ± 11^{a}	96 ± 4^{a}	257 ± 9^{a}	17 ± 3^{a}	304 ± 8^{a}	31 ± 4^{a}	115 ± 2^{a}
Enzymatic extraction	295 ± 20^{b}	116 ± 10^{b}	164 ± 26^{b}	44 ± 8^{b}	308 ± 15^{a}	47 ± 6^{b}	121 ± 5^{a}
Whole grain rye flour							
Free vitamins	246 ± 6^{a}	82 ± 6^{a}	289 ± 33^{a}	50 ± 1^{a}	241 ± 10^{a}	32 ± 7^{a}	92 ± 2^{a}
Enzymatic extraction	246 ± 13^{a}	91 ± 5^{a}	191 ± 14^{a}	65 ± 4^{b}	234 ± 11^{a}	41 ± 4^{a}	105 ± 2^{b}
Dark rye flour							
Free vitamins	236 ± 8^{a}	67 ± 3^{a}	185 ± 26^{a}	31 ± 3^{a}	174 ± 5^{a}	26 ± 4^{a}	75 ± 2^{a}
Enzymatic extraction	261 ± 4^{b}	90 ± 3^{b}	136 ± 11^{b}	39 ± 6^{a}	165 ± 16^{a}	35 ± 3^{b}	83 ± 3^{b}
Fine rye flour							
Free vitamins	185 ± 5^{a}	50 ± 2^{a}	144 ± 6^{a}	26 ± 2^{a}	122 ± 2^{a}	17 ± 4^{a}	49 ± 2^{a}
Enzymatic extraction	186 ± 5^{a}	58 ± 1^{b}	98 ± 15^{b}	40 ± 8^{b}	114 ± 4^{b}	23 ± 5^{b}	49 ± 4^{a}
Wheat flour							
Free vitamins	185 ± 2^{a}	37 ± 3^{a}	140 ± 16^{a}	18 ± 1^{a}	198 ± 3^{a}	19 ± 4^{a}	53 ± 3^{a}
Enzymatic extraction	185 ± 6^{a}	NA	101 ± 26^{b}	29 ± 3^{b}	196 ± 12^{a}	30 ± 1^{b}	57 ± 5^{a}
White rye malt							
Free vitamins	48 ± 2^{a}	147 ± 5^{a}	649 ± 146^{a}	0^{a}	241 ± 29^{a}	31 ± 6^{a}	80 ± 3^{a}
Enzymatic extraction	177 ± 7^{b}	193 ± 8^{b}	460 ± 25^{b}	64 ± 5^{b}	276 ± 10^{a}	62 ± 6^{b}	120 ± 5^{b}
Red rye malt							
Free vitamins	86 ± 27^{a}	112 ± 4^{a}	1138 ± 61^{a}	175 ± 7^{a}	NA	ND	49 ± 2^{a}
Enzymatic extraction	94 ± 9^{a}	110 ± 1^{a}	1176 ± 109^{a}	146 ± 21^{a}	NA	ND	117 ± 7^{b}
Yeast							
Free vitamins	2869 ± 80^{a}	1334 ± 27^{a}	4855 ± 76^{a}	1718 ± 21^{a}	5942 ± 135^{a}	198 ± 25^{a}	25 ± 1^{a}
Enzymatic extraction	4455 ± 719^{b}	2991 ± 218^{b}	9003 ± 113^{b}	3326 ± 11^{b}	8765 ± 91^{b}	214 ± 41^{a}	15 ± 1^{b}
Rye bread							
Free vitamins	129 ± 6^{a} *	50 ± 2^{a} *	210 ± 16^{a}	431 ± 31^{a} *	151 ± 9^{a}	10 ± 1^{a} *	42 ± 1^{a} *
Enzymatic extraction	139 ± 5^{a*}	89 ± 2^{b}	117 ± 24^{b}	438 ± 31^{a*}	168 ± 13^{a}	11 ± 2^{a*}	73 ± 2^{b}
Free vitamins, calculated	209 ± 7	65 ± 3	200 ± 23	35 ± 2	168 ± 4	23 ± 3	66 ± 2
Enzymatic extraction calculated	233 ± 5	85 ± 3	156 ± 10	41 ± 5	162 ± 13	31 ± 3	74 ± 2
Fine rye bread							
Free vitamins	98 ± 3^{a*}	30 ± 1^{a*}	88 ± 10^{a*}	502 ± 9^{a*}	132 ± 4^{a*}	8 ± 1^{a*}	24 ± 2^{a*}
Enzymatic extraction	140 ± 6^{b*}	82 ± 5^{b*}	57 ± 5^{b*}	535 ± 48^{a*}	135 ± 8^{a}	14 ± 2^{b*}	43 ± 3^{b}
Free vitamins, calculated	176 ± 2	49 ± 1	151 ± 15	26 ± 1	142 ± 3	17 ± 1	48 ± 1
Enzymatic extraction calculated	181 ± 2	165 ± 1	108 ± 4	39 ± 1	136 ± 3	24 ± 1	50 ± 1
Irradiated rye flour without *L. panis* N915, 0 h							
Free vitamins	199 ± 54^{a}	50 ± 4^{a}	259 ± 16^{a}	9 ± 2^{a}	396 ± 78^{a}	38 ± 9^{a}	100 ± 14^{a}
Enzymatic extraction	218 ± 7^{a}	50 ± 3^{a}	164 ± 6^{b}	0^{b}	349 ± 37^{a}	55 ± 4^{a}	94 ± 4^{a}
Irradiated rye flour without *L. panis* N915, 24 h							
Free vitamins	259 ± 17^{a}	45 ± 2^{a}	479 ± 55^{a}	19 ± 3^{a}	559 ± 40^{a}	32 ± 4^{a}	97 ± 8^{a}
Enzymatic extraction	246 ± 5^{a}	45 ± 2^{a}	344 ± 23^{b}	11 ± 3^{b}	520 ± 34^{a}	40 ± 8^{a}	101 ± 3^{a}
Irradiated rye flour with *L. panis* N915, 24 h							
Free vitamins	242 ± 5^{a}	46 ± 5^{a}	80 ± 1^{a}	613 ± 39^{a}	223 ± 6^{a}	31 ± 6^{a}	88 ± 4^{a}
Enzymatic extraction	225 ± 6^{b}	134 ± 35^{b}	56 ± 16^{a}	386 ± 13^{b}	215 ± 13^{a}	31 ± 3^{a}	96 ± 5^{a}

NA – Not available ND – Not detected.
Mean values of the vitamins in flours or rye bread with and without enzymatic treatment followed by a different superscript letter are significantly different at $p < 0.05$.
* – experimental value is different from calculated value ($p < 0.05$).

treatment with an enzyme mixture containing β-glucosidase from almonds did not increase pyridoxal and pyridoxine concentration in the reference flour. The concentration of pyridoxamine could not be adequately determined due to its very low retention under the chromatography conditions used and low concentration.

For vitamins B2, B3 and B5 the certificated values for standard material BCR 121 were not available and thus could not be compared with our results.

The concentration of thiamine was the highest in steel cut rye, whole grain rye flour and dark rye flour, remaining in the range of 235–246 μg/100 g DM. Fine rye flour and wheat flour contained less free thiamine (185 μg/100 g DM). In red rye malt and white rye malt, the concentration of thiamine (85 μg/100 g DM and 47 μg/100 g DM, respectively) was significantly lower than in whole grain rye flour.

Adding the enzymes to the extraction medium increased the concentration of thiamine up to 30%. The effect of enzymes was statistically significant for steel cut rye, dark rye flour, white rye malt, fine rye bread; however, it was not statistically significant for whole grain rye flour, fine rye flour, red rye malt, and rye bread.

Notably, the expected concentration of thiamine in rye bread (calculated based on the recipe and the respective thiamine content in the ingredients used) was 60% higher than that determined experimentally.

The concentration of riboflavin in whole grain rye flour and steel cut rye remained 82–96 μg/100 g DM, 67 μg/100 g DM in dark rye flour, 50 μg/100 g DM in fine rye flour and 40 μg/100 g DM in wheat flour. Enzymatic extraction increased the concentration of riboflavin significantly (10–35%) in both rye flour and rye bread samples. Notably, in wheat flour it was not possible to accurately measure the concentration of riboflavin using enzymatic extraction because of interfering peptides. The concentration of riboflavin in the rye bread was 50 μg/100 g without and 89 μg/100 g with enzymatic extraction. The theoretical riboflavin concentration, predicted on the basis of recipe, in rye bread was 30% higher than the experimentally measured concentration.

The concentration of pantothenic acid in steel cut rye (about 300 μg/100 g DM) was higher than in rye flours (122–241 μg/100 g DM). The enzyme extraction of samples had no effect on pantothenic acid concentration. The concentration of pantothenic acid calculated from the recipe was similar to that determined experimentally.

Similar to the results obtained on the reference sample, the sum of pyridoxal and pyridoxine in the flours and breads was lower than has been reported in the literature (Table 4). The concentration of pyridoxal in flours and rye breads was 8–31 μg/100 g DM and that of pyridoxine was 24–115 μg/100 g DM. Applying enzymatic treatment increased the concentration of pyridoxal and pyridoxine in the flour samples to some extent, most likely due to the dephosphorylation of corresponding phosphates. For malt samples, the enzymatic treatment had a more pronounced effect (up to 50–60%). Recipe based contents of pyridoxine and pyridoxal in rye bread were 36–59% higher than those determined experimentally, suggesting that both vitamers were unstable during processing.

The concentration of nicotinic acid and nicotinamide in whole grain rye flour and steel cut rye were about 250–300 and 15–50 in μg/100 g DM, respectively. Notably, while the concentration of nicotinamide increased with enzyme treatment, the concentration of nicotinic acid decreased by a considerable extent. The concentration of nicotinic acid in white and red rye malt was significantly higher than that in flours: 650 and 1140 μg/100 g DM, respectively. This can be explained by the increase of nicotinic acid concentration during malting. Interestingly, after rye bread processing the nicotinamide concentration (430 μg/100 g DM) was significantly higher than the theoretically calculated value (34 μg/100 g DM), while the concentration of nicotinic acid did not change significantly. This increase in the concentration of nicotinamide observed

Table 4
The concentration of B-complex vitamins and dietary fiber in flours and breads from different data sources.

Name	Reference	B1	B2	B3	B5	B6	Insoluble DF	Soluble DF	Total DF
		μg/100 g of edible portion					g/100 g of edible portion		
Rye									
Crushed rye grain	Finnish food composition database FINELI	280	100	ND	ND	280	18.1	2.2	18.1
Rye kernels	Danish Food Composition Databank	359	152	1680	1340	234	–	–	14.8
Rye	USDA Nutrient Database for Standard Reference	316	251	4270	1456	294	–	–	15.1
Dark rye flour							–	–	–
Dark rye flour	Finnish food composition database FINELI	300	200	400	–	160	10.8	2.1	9.4
85% extraction	Danish Food Composition Databank	330	82	–	430	–			10.5
Dark rye flour	USDA Nutrient Database for Standard Reference	316	251	4270	1456	443	–	–	23.8
Medium rye flour		287	114	1727	492	268	–	–	11.8
Fine rye flour									
70% extraction	Danish Food Composition Databank	234	49	–	370	–			4.4
Light rye flour	USDA Nutrient Database for Standard Reference	287	90	800	492	234	–	–	8
Rye bread									
Rye bread, 51% rye	Finnish food composition database FINELI	150	120	600	–	–	6.5	1.4	8.1
Rye bread		190	150	400	–	–	8.5	1.6	11.0
Rye dark bread	Danish Food Composition Databank	191	69	850	600	161	–	–	8.6
Rye dark, wholemeal bread		110	54	470	600	130	–	–	6.1
Rye bread	USDA Nutrient Database for Standard Reference	434	335	3805	440	75	–	–	5.8

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during rye bread processing can be attributed to lactic acid fermentation.

Notably, compared to the 10 kGy irradiated control sample without LAB the concentration of nicotinamide increased during the fermentation of the flour by lactic acid bacteria (*L. panis* N915) nearly 60 times and that of nicotinic acid and pantothenic acid decreased about 3 and 1.7 times, respectively (Table 3). Such changes were not observed during lactic acid fermentation for the other vitamers.

4. Discussion

In sourdough fine rye breads, the experimentally measured total dietary fiber was higher than that calculated on the basis of the raw materials and the bread recipe. The increase in total dietary fiber during bread processing has been observed previously. Johansson et al. (1984) observed the increase of total dietary fiber content in wheat and rye breads made from low extraction flour and found that this was caused by the formation of resistant starch during baking. The increase in soluble dietary fiber content during bread processing observed in this work can be explained by the partial conversion of insoluble fiber into soluble during rye flour fermentation, scalding, as well as during leavening with yeast. Such redistribution of dietary fiber forms by the activities of intrinsic enzymes in rye flour (α − amylase, β − xylosidase, α − arabinofuranosidase, β-glucanase, endo-xylanase and cinnamoyl esterase) was also suggested by Boskov Hansen et al. (2002).

The decrease in the concentration of specific vitamers during rye sourdough bread processing was significant for thiamine, riboflavin and pyridoxine. In general, the loss of those vitamers during processing, much like degradation at higher temperatures applied during baking, was similar to that observed earlier (Batifoulier et al., 2005, 2006; Martinez-Villaluenga et al., 2009). The lower concentration of thiamine in the red and white rye malt than in grain can be explained by losses of thiamine during the malt production processes, where kilning at high temperatures with air-flow and removal of dried sprouts reduce its content remarkably.

The nutritional databases report significantly higher vitamin B3, B5 and B6 content in cereals than were determined in this work (Tables 3 and 4). This could be due to various reasons. First, the data in the databases derives from several sources and is usually analyzed by different extraction and analysis methods. For example, Kall (2003) showed that vitamin B6 content in foods obtained with a microbiological assay and HPLC was systematically different and the variance of the results depended mainly on the extraction method and the sample matrix. Second, grains from different geographic locations may vary in vitamer content.

In order to determine whether vitamin content determined in the present work for the cereal products was affected by insufficient or unsuitable enzymatic activities of the enzymes in the extraction solution, we studied the effect of enzyme preparations: α-amylase, β-glucosidase, and acid phosphatase on the recovery of thiamine, riboflavin, nicotinamide and pantothenic acid from their different cofactors: TMP, TDP, FMN, FAD, NAD^+, NADH, and CoA (Table 5). More than 90% recovery of thiamine (from TMP and TDP) and riboflavin (from FMN) was observed in case of all enzyme preparations, suggesting their sufficient acid phosphatase and acid pyrophosphatase activities. The α-amylase also had significant proteolytic activity which could cause overestimation of riboflavin in the case of flour samples (including the reference sample) due to formation of peptides with similar *m/z* to that of riboflavin. This was, in particular, the case for wheat flour riboflavin analysis where the α-amylase treatment increased the riboflavin content by 20 fold. We have modeled the formation of possible peptide fragments with $[M + H]^+ = 377.15 +/- 0.5$ Da from various wheat proteins (gliadins, glutenins, avenin-like proteins) using the UniProt program (www.uniprot.org) and ExPASy Bioformatics Resources Portal (www.expasy.com) and analyzed their hydrophobicity and retention time in a C18 reversed phase column. As a result, several tetrapeptides, such as TSAV ($[M + H]^+$ 377.203), LSAS ($[M + H]^+$ 377.203), ISGT ($[M + H]^+$ 377.203), VCGV ($[M + H]^+$ 377.18), STGL ($[M + H]^+$ 377.203), and tripeptide QVM ($[M + H]^+$ 377.185) were shown to be possible interfering compounds for riboflavin analysis.

The recovery of nicotinic acid and nicotinamide from NAD^+ and NADH was less than 10%, even with treatment by enzyme preparations. This can be explained by the absence of purine nucleosidase activities in the enzyme preparations used. This was also strongly supported by the increased intensities of N-ribosyl nicotinamide peaks, observed in the mass spectrum of β-glucosidase and α-amylase treated NAD^+. Interestingly, instead of N-ribosyl nicotinamide, N-ribosyl nicotinic acid was observed in the mass spectra of NAD^+ treated with acid phosphatase (Table 5). N-ribosylnicotinic acid was also found in enzymatic extracts of the cereal samples. This observation could be evidence that our enzyme preparations exhibit potato nicotinamide riboside deaminase activity, which catalyzes the conversion of nicotinamide riboside

Table 5
Enzymatic activities and molar recovery (%) of vitamer subunits from Co-factors.

	Acid phosphatase (0.12 mg/mL)	β-Glucosidase (1.2 mg/mL)	α-Amylase (5.0 mg/mL)	0.1 N HCl 121 °C
Proteolytic activity[a]	50 U/mg	0.12 U/mg	2804 U/mg	–
Thiamine recovery, %				
From TMP	101	88	95	10
From TPP	96	105	105	9
Riboflavin recovery, %				
From FMN	98	102	92	6
From FAD	93	106	95	7
Nicotinamide recovery, %				
From NAD	0	9	10	91
From NADH	6	4	13	6
Nicotinamide riboside recovery, %				
From NAD	+(Nicotinic acid riboside)	+	+	ND
From NADH	+(Reduced form of nicotinic acid riboside)	+(Reduced form)	+(Reduced form)	ND
Pantothenic acid recovery, %				
From Coenzyme-A	0	0	0	0
Pantetheine recovery, %				
From Coenzyme-A	+	+	+	NT

+ − identified in the mass spectra by the *m/z* ratio.
ND − not detected; NT − not tested.
[a] Protease (side) activity in the enzyme preparations used (expressed as BAEE units of trypsin).

into nicotinic acid riboside (Katahira and Ashihara, 2009). Unfortunately, due to the unavailability of nicotinamide and nicotinic acid riboside standards, the recovery values of this vitamer from NAD^+ could not be determined. Acid treatment (0.1 M HCl, 121 °C, 30 min) liberated 90% of nicotinamide from NAD^+ by cleavage of the N-glycosidic bond. This can explain the higher values for niacin reported in the literature since the standard method usually involves the extraction in 0.1 M HCl at 100 °C for 60 min (EN 14663:2006). On the other hand, the ability of human GI to utilize NAD^+ as vitamin B3 is unclear.

The concentration of nicotinamide increased with lactic acid fermentation of the rye dough while that of nicotinic acid decreased. This process cannot be related to direct conversion of nicotinic acid into nicotinamide because this reaction is thermodynamically irreversible under physiological conditions. According to the KEGG metabolic pathways (www.kegg.com), nicotinic acid (and NAD^+) can be converted into nicotinamide by lactobacilli over a series of enzymatic reactions. This microbial conversion, as well as the acidic conversion of NAD^+ into B3 vitamers in the gastrointestinal tract (GIT) requires a separate study.

Pantothenic acid as a chemical compound could not be recovered by enzymatic treatment from Coenzyme A. However, all enzyme preparations resulted in liberation of pantetheine, which has been shown to have vitamin B5 activity (Aguilar et al., 2008). Again, due to the unavailability of isotope labeled pantetheine, the vitamer recovery could not be taken into account which, in turn, may have caused the underestimation of vitamin B5 (pantothenic acid) content in our study.

The concentration of pyridoxine + pyridoxal determined in our work was more than 40% lower than the B6 content reported in databases as well as for the reference material. The effect cannot be explained by the fact that pyridoxamine could not be quantified under the chromatographic conditions used because the content of pyridoxamine in flour has been reported to be only 10–12% of the total B6 content (Polansky and Toepfer, 1971). A more plausible explanation for lower B6 content is poor hydrolysis of glycosylated pyridoxine. The latter is common in grains and may account for as much as 56% of total B6 in wheat cultivars (Sampson et al., 1995). It is possible that, in contrast to the standard methods (EN 14663:2006) which use the HCl extraction at 121 °C, the enzyme mixture used in this work was unable to convert 5′-O-β-D-glucopyranosyl pyridoxine into pyridoxine. Notably, the pyridoxine glucoside serves as a source of nutritionally available vitamin B6, with ~50% bioavailability relative to pyridoxine (Nakano et al., 1997).

5. Conclusion

Our results demonstrate that theoretical concentrations of dietary fiber and vitamers calculated on the basis of raw ingredients can differ significantly from concentrations measured in baked rye bread. We attribute this difference to biochemical and microbiological processes that occur during bread making.

The LC–MS method we employ, combined with the stable isotope dilution assay, is a promising method both for the simultaneous analysis of B-complex vitamins, and for studying their stability during food processing. However, for a complete analysis of the B-group vitamins in food using this method, additional labeled vitamers or their precursors should be incorporated in the internal standard solutions and/or extraction steps should be further optimized.

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References

Aguilar, F., Charrondiere, U.R., Dusemund, B., Galtier, P., Gilbert, J., Gott, D.M., Grilli, S., Guertler, R., Kass, G.E.N., Koenig, J., Lambré, C., Larsen, J.-C., Leblanc, J.-C., Mortensen, A., Parent-Massin, D., Pratt, I., Rietjens, I., Stankovic, I., Tobback, P., Verguieva, T., Woutersen, R., 2008. Pantethine as source for pantothenic acid added as a nutritional substance in food supplements. The EFSA Journal 865, 1–20.

AOAC, 2000. Official Methods of Analysis of AOAC International. Method 923.03. Determination of Ash. Gravimetric Method. AOAC International, USA.

Aura, A.-M., Myllymäki, O., Bailey, M., Penalvo, J.-L., Adlercreutz, H., Poutanen, K., 2007. Interrelationships between carbohydrate type, phenolic acid and initial pH on in vitro conversion of enterolactone from rye lignans. In: Salovaara, H., Gates, F., Tenkanen, M. (Eds.), Dietary Fibre Components and Functions. Waageningen Academic Publishers, The Netherlands, pp. 235–245.

Axelsson, L., 2007. Lactic acid bacteria: classification and physiology (Chapter 1). In: Salminen, S., von Wright, A., Ouwehand, A. (Eds.), Lactic Acid Bacteria. Microbiological and Functional Aspects. Markel Dekker, Inc, NY, pp. 1–67.

Batifoulier, F., Verny, M.-A., Chanliaud, E., Remesy, C., Demigne, C., 2005. Effect of different breadmaking methods on thiamine, riboflavin and pyridoxine contents of wheat bread. Journal of Cereal Science 42, 101–108.

Batifoulier, F., Verny, M.-A., Chanliaud, E., Remesy, C., Demigne, C., 2006. Variability of B vitamin concentrations in wheat grain, milling fractions and bread products. European Journal of Agronomy 25, 163–169.

Boskov Hansen, H., Andreasen, M.F., Nielsen, M.M., Larsen, L.M., Bach Knudsen, K.E., Meyer, A.S., Christensen, L.P., Hansen, A., 2002. Changes in dietary fibre, phenolic acids and activity of endogenous enzymes during rye bread-making. European Food Research and Technology 214, 33–42.

Buchholz, M., Drotleff, A.M., Ternes, W., 2011. Thiamin (vitamin B1) and thiamin phosphate esters in five cereal grains during maturation. Journal of Cereal Science 56, 109–114.

Cataldi, T.R.I., Nardiello, D., De Benedetto, G.E., Bufo, S.A., 2002. Optimizing separation conditions for riboflavin, flavin mononucleotide and flavin adenine dinucleotide in capillary zone electrophoresis with laser-induced fluorescence detection. Journal of Chromatography A 968, 229–239.

Elleuch, M., Bedigian, D., Roiseuz, O., Besbes, S., Blecker, C., Attia, H., 2011. Dietary fibre and fibre-rich by-products of food processing: characterisation, technological functionality and commercial applications: a review. Food Chemistry 124, 411–421.

EN 14663, 2006. Foodstuffs – Determination of Vitamin B6 (Including Its Glycosylated Forms) by HPLC. The European Standardisation Committee, Brussels.

ExPASy Bioformatics Resources Portal. Swiss Institute of Bioinformatics. Retrieved February 10, 2012 from ExPASy Bioformatics Resources Portal Homepage: www.expasy.com

Hansen, H.B., Rasmussen, C.V., Bach Knudsen, K.E., Hansen, A., 2003. Effect of genotype and harvest year on content and composition of dietary fibre in rye (*Secale cereale* L.) grain. Journal of the Science of Food and Agriculture 83, 76–85.

Hauf, J., Zimmermann, F.K., Müller, S., 2000. Simultaneous genomic overexpression of seven glycolytic enzymes in the yeast *Saccharomyces serevisiae*. Enzyme and Microbiol Technology 26, 688–698.

Johansson, C.-G., Siljeström, M., Asp, N.-G., 1984. Dietary fibre in bread and corresponding flours – formation of resistant starch during baking. Zeitschrift für Lebensmittel Untersuchung und Forschung A 179 (1), 24–28.

Kall, M.,A., 2003. Determination of total vitamin B6 in foods by isocratic HPLC: a comparison with microbiological analysis. Food Chemistry 82, 315–327.

Karppinen, S., Myllymäki, O., Forssell, P., Poutanen, K.F., 2003. Fructan content of rye and rye products. Cereal Chemistry 80 (2), 168–171.

Katahira, R., Ashihara, H., 2009. Profiles of the biosynthesis and metabolism of pyridine nucleotides in potatoes (*Solanum tuberosum* L.). Planta 231, 35–45.

KEGG – Kyoto Encyclopedia of Genes and Genomes, Release 58, Retrieved February 15, 2012 from the KEGG Pathway Database: www.genome.jp/kegg/pathway.html.

Lee, S.C., Prosky, L., DeVries, J.W., 1992. Determination of total, soluble, and insoluble dietary fiber in foods – enzymatic-gravimetric method, MES-TRIS buffer: collaborative study. Journal of the Association of Official Analytical Chemists 75, 395–416.

Leklem, J.E., 2001. Vitamin B6. In: Rucker, R.B., Suttie, J.W., McCormic, D.B., Machlin, L.J. (Eds.), Handbook of Vitamins, third ed. Marcel DeKker Inc, NY, pp. 339–396.

Liukkonen, K.-H., Heiniö, R.-L., Salmenkalloo-Marttila, M., Autio, K., Katina, K., Poutanen, K., 2006. Rye. In: Hui, Yiu H., Corke, Harold (Eds.), Bakery Products: Science and Technology. Blackwell Publishing, USA, pp. 109–122.

Martinez-Villaluenga, C., Michalska, A., Frias, J., Piskula, M.K., Vidal-Valverde, C., Zielinski, H., 2009. Effect of flour extraction rate and baking on thiamine and riboflavin content and antioxidant capacity of traditional rye bread. Journal of Food Science 74 (1), 49–55.

Mihhalevski, A., Sarand, I., Viiard, E., Salumets, A., Paalme, T., 2011. Growth characterization of individual rye sourdough bacteria by isothermal microcalorimetry. Journal of Applied Microbiology 110 (2), 529–540.

Nakano, H., McMahon, L.G., Gregory II, J.F., 1997. Pyridoxine-5′-b-D-glucoside exhibits incomplete bioavailability as a source of vitamin B-6 and partially inhibits the utilization of co-ingested pyridoxine in humans. The Journal of Nutrition 127 (8), 1508–1513.

Ottaway, P.B., 2002. The stability of vitamins during food processing. In: Henry, C.J.K., Chapman, C. (Eds.), The Nutritional Handbook for Food Processors. Woodhead Publishing in Food Science and Technology, UK, pp. 247–264.

Polansky, M.M., Toepfer, E.W., 1971. Effect of umigation on wheat in storage. Iii. Vitamin B-6 components of wheat and wheat products. Cereal Chemistry 48, 392–396.

Rindi, G., Laforenza, U., 2000. Thiamine intestinal transport and related issues: recent aspects. Proceedings of the Society for Experimental Biology and Medicine 224, 246–255.

Roth-Maier, D.A., Kettler, S.I., Kirchgessner, M., 2002. Availability of vitamin B6 from different food sources. International Journal of Food Sciences and Nutrition 53, 171–179.

Sampson, D.A., Eoff, L.A., Yan, X.L., Lorenz, K.L., 1995. Analysis of free and glycosylated vitamin B6 in wheat by high-performance liquid chromatography. Cereal Chemistry 72 (2), 217–221.

Uniprot – Universal Protein Resource.The UniProt Consortium, 2011. Ongoing and future developments at the universal protein resource. Nucleic Acids Research 39, D214–D219. Retrieved February 10, 2012 from UniProt Consortium Homepage. www.uniprot.com.

Wall, J.S., Carpenter, K.J., 1988. Variation in availability of niacin in grain products. Food Technology 42 (10), 198–204.

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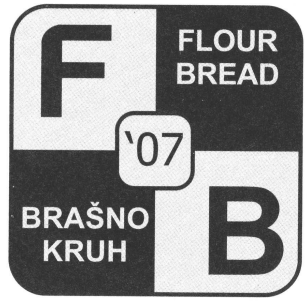

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APPLICATION OF ISOTHERMIC CALORYMETRY AND DYNAMIC VISCOSIMETRY IN STUDIES OF RYE BREAD STALING

UDC 664.662 : 539.501 + 536.6

***A. Mihhalevski*[1,2], *T. Paalme*[1,2]**

[1]*Competence Center of Food and Fermentation Technologies, Akadeemia tee 15, 12618, Tallinn, Estonia*

[2]*Department of Food Processing, Tallinn University of Technology, Ehitajate tee 5, 19086, Tallinn, Estonia*

ABSTRACT

The staling of bread is occurring due to the phase transitions causing the change in rheological properties and heat flow. The process can by continuously recorded using isothermal calorimetry and dynamic viscosimetry.

In this study the isothermal micro calorimeter TAM III (Thermometric, Sweden) was used to monitor the heat flow of rye bread, wheat bread and "micro-baked" dough and starch. Making micro-baked dough and starch preparations the process of baking was simulated in 3-ml vials in thermostat. Reaching storage temperature (20 or 35 °C) heat flow was recorded in micro calorimeter during two weeks at 20 °C. The initial heat flow (25-12 μW g^{-1} starch) decreased quickly during first 4-5 h of storage down to the level of 9-7, after which slow decrease in heat flow was observed.

In parallel the bread samples were studied rheologically during cooling and storage using rheometer Physica 301 MCR and texture profile analyzer TA-XT2. The results demonstrated that both heat flow (per gram of starch) and increased rate of visco-elastic module and hardness were higher for wheat bread than for rye bread.

Key words: rye bread, bread staling, bread texture, calorimetry, rheology

INTRODUCTION

During storage, bread continuously loses its freshness and stales. Migration of water and retrogradation of starch are most important factors for this process [1]. The staling of the bread is occurring due to the phase transitions causing the change in rheological properties and heat flow [2]. The main aim of this work was to develop the methods to study the dynamics of changes in viscoelastic and thermal properties of dough during simulations of bread baking and storage. The starches samples of similar to dough water content were studied in parallel.

MATERIALS AND METHODS

Materials

The fermented rye sour and wheat dough was derived from bakery "Leibur" (Estonia).

Rye starch was isolated from fine-grounded rye flour by alkaline extraction [3]. Wheat starch was obtained from Fluka. The starch water mixtures with water content (42%) similar to wheat and rye dough water content were prepared for simulations of baking process.

Analytical methods

Total starch in dough samples was determinated according to amyloglucosidase/ α-amylase method [4], water concentration was measured by Halogen Moisture Analyser HR 83 (Metler Toledo, USA) and activity by Water Activity Meter FA-st LAB (GBX, France). The dynamic viscoelasticity of dough and starch samples (42% of water) was measured using a rheometer Physica MCR 301 using 25 mm parallel plates (PP 25, gap 2 mm) by oscillation test at frequency 1 Hz and strain 1%. Thermal activity was measured TAM III Thermal Activity Monitor. Bread firmness was measured according AACC method 74-09 by Texture analyser TA-XT2i. Time layout of the measurements during baking and storage is shown on Figure 1.

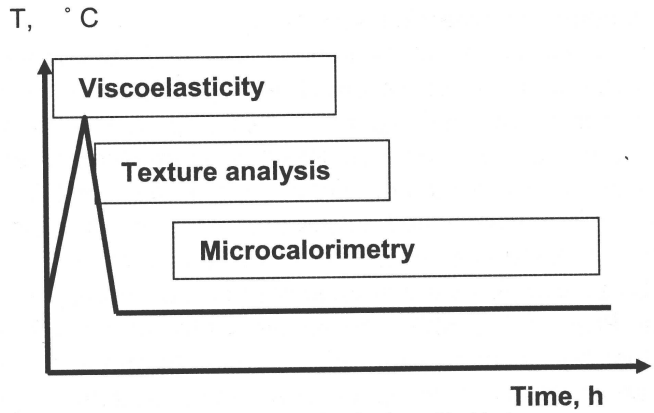

Figure 1. Time layout of the measurements after the beginning of baking

Simulations of baking and storage

The temperature profile in the middle of bread during baking process (see Figure 2) was simulated by micro-baking small amounts of dough or starch samples either 1) in 3 ml Thermal Activity Monitor vials before placing into the isothermal microcalorimeter or 2) directly between plates of Physica MCR 301 rheometer increasing temperature. Dough samples for texture analysis were proofed 40 min at 35 °C and baked at 220 °C 45 min in Clima Plus Combi (Metos HCPC).

RESULTS

Storage modulus profiles (Figure 2) during micro-baking of rye and wheat starch were slightly different. The maximum on the storage modulus G' curve occurred at 53.5C (267 kPa) for wheat and at 56 °C (178 kPa) for rye starch. The storage modulus profiles of rye and wheat dough micro-baking were very different. After reaching maximum baking temperature the wheat bread obtained 3 fold higher maximum

storage modulus value than the rye bread. Storage modules of both started to decrease during storage at 20 °C.

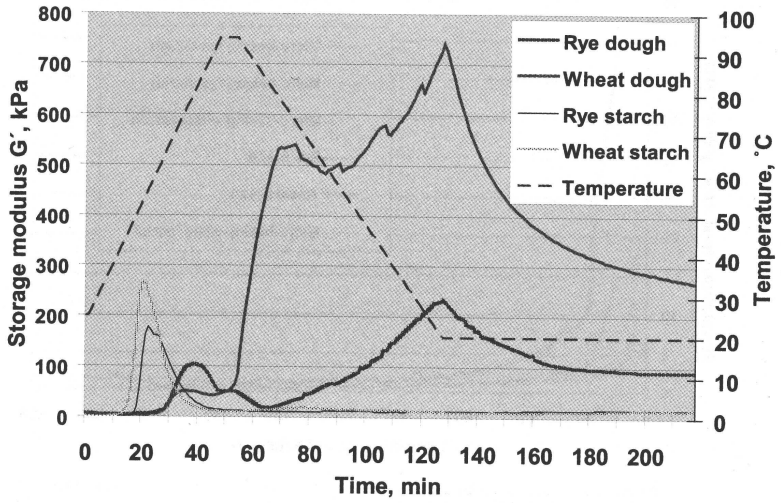

Figure 2. Elastic properties of dough and starch during baking and storage

Increase of crumb firmness after baking was measured during cooling and storage for both rye and wheat bread (Table 1). The firmness of rye bread was in all stages higher that of wheat bread. Increase in firmness during cooling and storage at 20 °C (from 13.41 to 54.34 N) was 4 fold for rye and 6.3 fold for wheat (from 1.94 to 12.21 N).

Table 1. The evaluation of sourdough rye bread and wheat bread after baking

Time of start of baking, min	Temperature, °C	Force, N Rye bread	Force, N Wheat bread
55	87	13.41	1.94
65	74	17.00	2.10
75	63	18.71	2.84
85	54	21.65	3.02
95	42	21.84	3.71
105	36	26.58	4.65
120	29	28.07	5.70
130	21	32.96	6.08
1170	20	44.33	7.78
2730	20	54.34	12.21
1170	20	44.33	7.78
2730	20	54.34	12.21

Thermal activity of micro-baked rye and wheat dough samples as well as rye and wheat starch samples was measured during 200 h at 20 °C (storage). Results shown in Figure 3A demonstrated that heat flow (μW per gram of starch in sample) was in case of rye starch and bread lower than in wheat starch and bread respectively. During

the first 20 h from the start of baking heat flow decrease of wheat starch (from 29 μW to 5 μW) is significantly higher and more rapid than that of rye starch (11 to 2 μW).

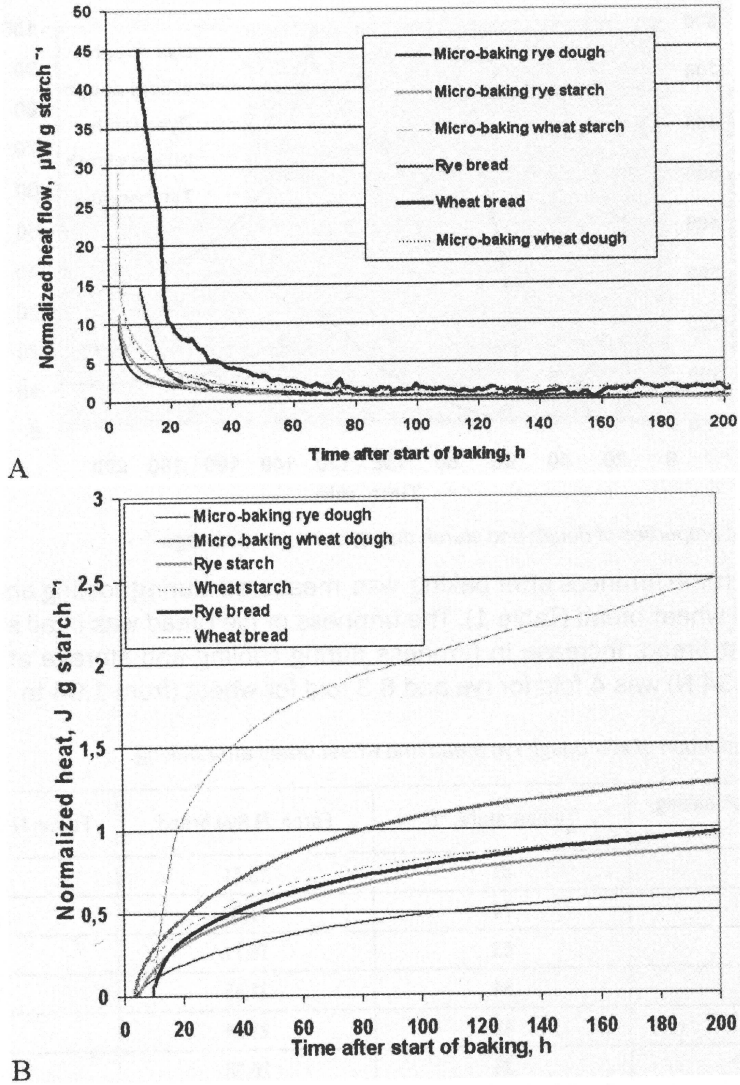

Figure 3. Heat flow (A) and heat production (B) after baking and micro-baking

CONCLUSIONS

The isothermal calorimetry and dynamic viscosimetry can be used for study of the staling process of bread.

Storage modulus G' of rye starch during baking process was lower than that of wheat starch. Storage modulus G' of dough samples started to increase significantly later those of starch.

- Wheat bread firmness increased during cooling and storage more rapidly (6.3 fold) than that of rye bread (4 fold).
- Thermal activity monitoring demonstrates that the heat flow values (W g starch^{-1})

of (micro-) baked dough are after cooling down to 20 °C significantly lower for rye dough than for wheat dough.

- The staling process in rye bread is slower than that of wheat bread.

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REFERENCES

1. Schiraldi, A., Fessas, D., Mechanism of Staling: An Overview. In: Bread staling (Eds. Chinachoti, P., Vodovotz, Y.), 2001, New York: CRC Press, pp. 1-17
2. Ribotta, D.P., Le Bail, A. Thermo-physical assessment of bread during staling. - Food Science and Technology, 2007, 40(5), 879-884.
3. Verwimp, T., Vandeputte, G.E., Marrant, K., Delcour, J.A. Isolation and characterisation of rye starch. – Journal of Cereal Science, 2004, 39(1), 85-90.
4. Total starch assay procedure (amyloglucosidase/α amylase method), 2006, KTSTA-05/06, Megazyme International Ireland Ltd., Ireland.

PUBLICATION VI

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Structural Changes of Starch during Baking and Staling of Rye Bread

Anna Mihhalevski,*[,†,□] Ivo Heinmaa,[‡] Rainer Traksmaa,[△] Tõnis Pehk,[‡] Arvo Mere,[○] and Toomas Paalme[†,□]

[†]Competence Center of Food and Fermentation Technologies, Akadeemia tee 15, 12618 Tallinn, Estonia

[□]Department of Food Processing, Tallinn University of Technology, Ehitajate tee 5, 19086 Tallinn, Estonia

[‡]National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 12618 Tallinn, Estonia

[△]Center of Material Research, Tallinn University of Technology, Ehitajate tee 5, 19086 Tallinn, Estonia

[○]Institute of Material Science, Tallinn University of Technology, Ehitajate tee 5, 19086 Tallinn, Estonia

ABSTRACT: Rye sourdough breads go stale more slowly than wheat breads. To understand the peculiarities of bread staling, rye sourdough bread, wheat bread, and a number of starches were studied using wide-angle X-ray diffraction, nuclear magnetic resonance (^{13}C CP MAS NMR, ^{1}H NMR, ^{31}P NMR), polarized light microscopy, rheological methods, microcalorimetry, and measurement of water activity. The degree of crystallinity of starch in breads decreased with hydration and baking to 3% and increased during 11 days of storage to 21% in rye sourdough bread and to 26% in wheat bread. ^{13}C NMR spectra show that the chemical structures of rye and wheat amylopectin and amylose contents are very similar; differences were found in the starch phospholipid fraction characterized by ^{31}P NMR. The ^{13}C CP MAS NMR spectra demonstrate that starch in rye sourdough breads crystallize in different forms than in wheat bread. It is proposed that different proportions of water incorporation into the crystalline structure of starch during staling and changes in starch fine structure cause the different rates of staling of rye and wheat bread.

KEYWORDS: *rye sourdough bread staling, starch retrogradation, amylose, amylopectin, XRD, MAS NMR*

INTRODUCTION

Rye sourdough bread is a traditional and popular bread in northwestern Russia, the Baltic states, Finland, northern Germany, and Denmark. In the Baltic states, rye bread is industrially produced by mixing sourdough with flour, baker's yeast, and scalded flour, followed by leavening, molding, proofing, and baking. Rye bread is a recommended dietary product because it is a good source of nutritionally important substances, including B-complex vitamins and dietary fiber. Complex technology and relatively short storage time causes the limitation of consumption of rye bread outside the traditional areas of production.

The main factor that limits the storage time of breads is staling, which is mainly a physical process in bread and other starch-containing foods that reduces their palatability. Staling is not simply a drying-out process due to evaporation; packing bread hermetically does not prevent staling. Although the precise mechanisms of staling remain unknown, most theories are related to migration of moisture within the microstructures of bread and structural changes in starch structure during storage. During baking the temperature inside the bread reaches 99 °C, during which starch granules swell, gelatinize, and partly liquidize.[1] After cooling, the crystalline structure of starch during storage slowly recovers.[2] This process is termed starch retrogradation. Retrogradation is technologically important because it produces significant changes in properties such as springiness, softness, and moistness of the crumb, which are important in the sensory perception of bread.[3] The rate of bread staling depends on the recipe used and the storage conditions, mainly temperature and humidity. During storage, water migration from the crumb to crust occurs, which leads to a decrease in water concentration and activity in the crumb[4] and softening of the crust.[5] However, water migration is not the main reason for bread staling and starch retrogradation. It is supposed that staling is caused mainly by retrogradation of amylopectin.[6] In starch, the short-chain nonbranched fraction of amylopectin molecules is organized as small crystallites formed from double helices.[7,8]

Linear molecules of amylase are apparently present in an amorphous state in the starch granule.[9,10] Amylopectin chains are primarily responsible for the crystallinity of starch. It was suggested that the branching points and intercluster connections of amylopectin are located in the more amorphous region.[11]

The crystalline structure of starch has been classified according to X-ray diffraction patterns as A-, B-, or C-type.[9] Typical cereal starches such as wheat and rye starches belong to the A-type, in which crystals contain double helices that are densely packed in a monoclinic lattice. The B-polymorph, characteristic of potato starch, has double helices packed in a hexagonal lattice with 27% hydration.[12,13] Mixed C-type starch is observed in legume starches.[2]

Also, starch−protein complexes are considered to be very important in the development of the firmness of the crumb during staling.[14] In contrast to wheat gluten, the rye proteins (secalins, prolamins) cannot take part in the formation of

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dough structure as a starch−gluten complex due to the lack of low molecular weight glutenin subunits, and it is thus impossible for intermolecular disulfide bonds to form.[15] In the case of rye bread, water-extractable arabinoxylans play an important structural role, binding water and forming viscous dough.[16,17] The low pH of rye sourdough increases the extractability and swelling properties of arabinoxylans and inactivates amylase activity. The processes mentioned above contribute to an increase in water-binding capacity.[18] These peculiarities might be why rye bread prepared with sourdough stales more slowly than wheat breads.[19]

Also, scalding, a common procedure during bread processing in several countries, might affect the rate of staling. Scalding is the mixture of flour and hot water, which is allowed to cool.[6] During scalding the starch is gelatinized and hydrolyzed partly by endogenous enzymes or added malt. Siljeström and coauthors[20] showed that retrogradation of starch in bread made from malted whole grain wheat flour was slower that that in bread made from unmalted whole grain wheat flour.

The aim of this work is to study and characterize the structural changes of starch in rye sourdough bread during baking and staling by comparing rye and wheat starch as well as bread structures during processing.

MATERIALS AND METHODS

Dough Ingredients. Lactic acid bacteria strains *Lactobacillus plantarum* L-73 and *Lactobacillus brevis* L-62 (freeze-dried) were kindly provided by Lallemand Inc. (Montreal, Canada) and used for the production of sourdough. Fresh baker's yeast (sp. *Saccharomyces cerevisiae*), used for bread dough leavening, was obtained from a local market.

Dark rye flour (type 1370) and wheat flour (W550) were obtained from the Tartu Grain Mill Ltd. (Estonia). Fermented (red) and unfermented (white) rye malts were obtained from Eesti leivalinnase Ltd. (Võru, Estonia). Sugar, margarine, milk powder, and salt were obtained from a local market.

Starches. Wheat, corn, and potato starches, amylose from potatoes, and amylopectin from maize were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Dimethyl sulfoxide (DMSO-d_6) was also obtained from Sigma-Aldrich.

Rye starch was isolated from fine-ground rye flour by alkaline extraction.[21] Rye flour was suspended in tap water and 0.5% NaOH in a ratio of 2:7:7, stirred for 60 min, and centrifuged at 3000 rpm for 10 min. The sediment was washed with water and centrifuged again. The procedure was repeated two times, neutralized with 0.1 N HCl, and centrifuged. The upper grayish layer was removed, and the white starch was washed with water and centrifuged again. The isolated starch was air-dried overnight and sieved trough a 250 μm sieve.

Baking. Rye sourdough breads were prepared in a laboratory from sourdough starter culture, rye flour, scalded flour, and yeast suspension. Sourdough was prepared according to method described by Mihhalevski and coauthors[22] by mixing 225 g of the dark rye flour and a bacterial suspension made from a mixture of *L. brevis* L-62 and *L. plantarum* L-73 (in a ratio of 1:1, 5×10^6−5×10^7 cfu mL^{-1}) in 0.5% of NaCl to a 1:1 ratio in Stomacher 400 circulator (Seward Ltd., UK) bags for 15 min at 100 rpm and then incubated in the stomacher bags in an Environmental Test Chamber (Sanyo, Japan) at 32 °C during 24 h.

Scalded flour was made by mixing 239.5 g of rye dark flour and 16.5 g of red rye malt into 810 mL of warm (55 °C) tap water in a 2 L temperature-controlled kettle equipped with a refrigerated/heating circulator (Julabo F25, Seelbach, Germany). After 15 min of mixing, rye white malt was added. This mixture was heated to 67 °C, and after saccharification during 40 min, it was cooled to 27 °C.

Sourdough (450 g) was mixed with scalded flour (1100 g), rye dark flour (1200 g), 30 g of yeast suspension (20% dry weight), and 120 g of 24% salt solution during 20 min in a 5 L Bear Teddy dough mixer (Varimixer, Shreveport, LA, USA) at 100 rpm. Dough fermentation occurred in the Environmental Test Chamber at 32 °C during 110 min. Dough was molded into 400 g portions, put into baking molds (8.5 × 15 × 9.5 cm), proofed in the Environmental Test Chamber at 32 °C during 45 min, and baked in a Self Cooking Center (Metos System Rational, Weikersheim, Germany) as follows: 10 min, 230 °C; 15 min, 200 °C; 15 min, 180 °C; 10 min, 150 °C. The bread was taken from the oven and cooled to room temperature in a laminar flow cabinet under UV light (Telstar, Terrassa, Spain), sealed aseptically into plastic bags, and stored at room temperature.

Wheat bread was made by mixing 50 mL of yeast suspension (20% DM) with sugar (14 g), wheat flour (300 g), margarine (10 g), milk powder (15 g), salt (5 g), and water (110 mL). Molded dough pieces were proofed for 20 min at 32 °C and baked for 30 min at 220 °C.

Microbaking. Starch−water or flour−water mixtures with 42% water content (similar to wheat and rye dough water content) were prepared for baking simulations. A small amount of dough or starch−water suspension was baked according to a temperature profile that simulates the baking profile inside the bread. The temperature profile was recorded during bread baking using a Data Logger (Onset, Cape Cod, MA, USA). Microbaking trials were carried out using three different methods: (i) in the 3 mL microcalorimeter vials in a thermostat; (ii) between plates (25 mm, gap of 2 mm) of a dynamic rheometer, Physica MCR 301 (Anton Paar, Ostfildern, Germany); (iii) between the object-plate and cover-glass, glued by silicone to hermetically seal the sample, in a thermostat VEB MLW U2C (Prüfgeräte-Werk, Medingen, Sitz Freital, Germany).

To determine the heat flows after baking, vials were placed into an isothermal microcalorimeter TAM III (TA Instruments, New Castle, DE, USA) at 20 °C for 200 h. The power−time (P−t) curves of samples were measured in 15 min intervals starting at 1 h after microbaking.

The storage modulus (G', kPa) of dough and starch samples during microbaking in the rheometer was measured using an oscillation test at a frequency of 1 Hz, a normal force of 1 N, and a strain of 0.01−10%.

Sample Preparation. Fresh-baked hot breads (0 h) were sliced in a laminar flow cabinet under UV light, packed aseptically in plastic bags, sealed hermetically, and stored at 22 °C and 95% relative humidity for 11 days for further analysis. After slicing at 0 h (immediately after removal from the oven) and storing for 5 h or 11 days, the bread samples were flash cooled in liquid nitrogen, freeze-dried, and ground (particle size = 0.25 mm).

The total starch in the dough and bread samples was determined using an amyloglucosidase/α-amylase assay kit from Megazyme International Ltd. (Bray, Ireland). Three milliliters of thermostable α-amylase (3% in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer, 50 mM, pH 7.0) and 200 μL of ethanol (80%) were added to sample (100 mg). The tube with mixture was placed in a boiling water bath for 6 min with stirring after 2 and 4 min. Sodium acetate buffer (4 mL, 200 mM, pH 4.5) and amyloglycosidase (100 μL) were added to the mixture, and the sample was incubated at 50 °C for 30 min. After incubation, the sample was diluted to 100 mL and centrifuged (3000 rpm, 10 min). After the addition of 10 μL of supernatant to 3 mL of glucose determination reagent (GOPOD) and incubation at 50 °C for 20 min, the mixture's absorbance was measured at 510 nm.

Moisture content was measured at 105 °C using a Halogen Moisture Analyzer HR 83 (Mettler Toledo, Urdorf, Switzerland). Water activity was measured using a Water Activity Meter FA-st LAB (GBX, Bourg de Peage, France).

XRD Analysis. The crystallinity of flours, starches, and freeze-dried and ground bread samples was studied by wide-angle X-ray diffraction (XRD) measurements using an X-ray diffractometer Ultima IV (Rigaku, Tokyo, Japan). The diffractometer settings were as follows: line detector D/tex Ultra, copper tube operating at 40 kV and 40 mA, and irradiation of the sample with Cu Kα radiation (1.541 Å) using a Ni filter to restrain Kβ radiation. Diffractograms were acquired at 25 °C over a 2θ range of 6−50° with a measurement speed of 5°/min. The step size was 0.02°.

The degree of crystallinity (DC, %) was determined according to method described by Ribotta and coauthors[23]

$$\text{DC, \%} = \frac{I_C}{I_C + I_A} \times 100 \quad (1)$$

where I_C is the integrated area of the crystalline phase and I_A is the integrated area of the amorphous phase. Data analysis was performed using the programs EVA and TOPAS 4.2 (Bruker).

NMR. ^{13}C CP MAS NMR spectra were recorded on a Bruker AVANCE II 600 MHz spectrometer using a 14.4 T external magnetic field and custom-built MAS probe for 4 mm rotors. The sample spinning speed was 15.0 kHz, and typically 10000 accumulations were averaged to obtain a reasonable signal-to-noise ratio. For all samples we applied a contact time of 0.5 ms for cross-polarization and a relaxation delay of 5 s between accumulations. All spectra were referenced to solid adamantane resonance lines at 29.46 and 38.48 ppm.[24]

^{1}H and ^{13}C NMR spectra of amylose (9 mg dissolved in 300 mg of DMSO-d_6) and amylopectin (7 mg dissolved in 300 mg of DMSO-d_6) model compounds were obtained in a Bruker AVANCE III 800 MHz spectrometer at a temperature of 313 K. To fit the obtained NMR spectra, the TOPSPIN program from Bruker was used.

^{31}P NMR spectra of starches dissolved in DMSO-d_6 were recorded on a Bruker AVANCE III 800 spectrometer at a frequency of 324 MHz (ambient temperature). H_3PO_4 (85%) resonance (at 0 ppm) and egg yolk phosphatidylcholine were used as external references.

Microscopy. Starch and dough samples were observed using a polarized light Nikon Eclipse E200 microscope (Tokyo, Japan) with 400× magnification and analyzed using the picture image analysis program ACT-2U. The micrographs were made before heating, after heating, and during storage for 7 days.

RESULTS

Microbaking. The effects of baking and storage on dough and starch water suspensions were studied using microbaking techniques. The contents of starch were 76.11 ± 1.01 g/100 g DM for wheat flour and 65.96 ± 1.77 g/100 g DM for rye flour.

Lenticular and spherical granules with a diameter of 2−40 μm were observed on microscope slides of hydrated rye starch, wheat starch, amylopectin, and rye dough (Figure 1). The rye starch granules were larger than those of amylopectin and wheat starch, observed also by Gomand and coauthors.[25] The form of a Maltese cross indicates orderly arrangement of the crystalline areas within each granule.[9] The pictures after heating to 99 °C showed losses of the Maltese cross pattern, which indicates that disordering processes related to starch gelatinization occurred. In the case of amylopectin isolated from maize, the structures containing Maltese cross formations were restored, whereas in the case of baked rye and wheat starch the longish structures appeared on the micrographs after cooling. During storage, the intensity of brightness of those structures increased, probably due to the crystallization processes. The longish structures were not observed on micrographs of pure amylopectin or amylose. The effect can be related to leakage of amylose from the starch granule. The increase in intensity of those structures from starch during storage might be related to the change of crystallinity and rheological properties during storage of bread. Hug-Iten and coauthors[26] suggested that the reorganization of the intragranular amylose fraction enhances the rigidity of starch granules on bread staling.

Changes in the viscoelastic properties of starches and dough were studied using baking between rheometer plates. The viscoelastic profiles of rye and wheat starch showed significant differences during the first stages (Figure 2). With an increase in temperature, the storage modulus (G') increase of pure wheat starch started at 40 °C and that of pure rye starch at 45 °C. With further increase of temperature over 55 °C the G' decreased. This is probably due to liquidation of the starch gel. In the case of wheat and rye dough, the increase in G' was

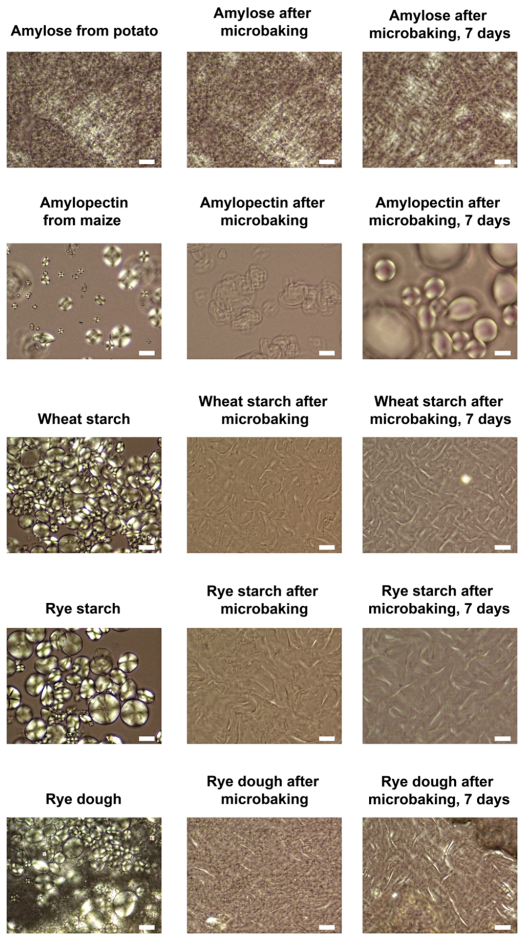

Figure 1. Polarized light micrographs of potato amylose−, maize amylopectin−, wheat starch−, rye starch−water suspensions and rye dough before and after microbaking and storage. Bar = 20 μm.

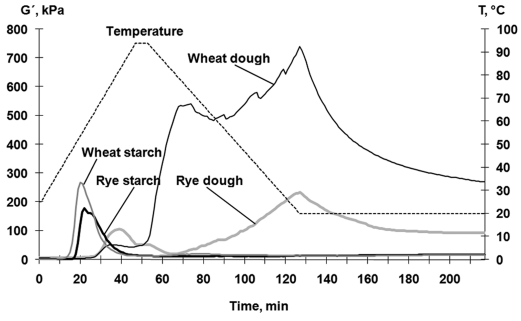

Figure 2. Viscoelastic properties of starch−water suspensions and dough during baking and cooling between rheometer plates.

observed at temperatures around 65 °C, whereas the storage modulus of pure starch decreased. The difference can be related to the effects of the dough matrix protecting starch from hydration and damage.

The behavior of wheat and rye dough was different during holding at 95 °C. In the case of rye dough the G' rather decreased, whereas in the case of wheat dough G' increased to 550 kPa. The difference may be related to the formation of a gluten network in wheat dough. Upon further cooling from 80 to 20 °C the G' increased for both doughs to equal extent, about 250 kPa. The process can be related to gel formation from liquidized starch, protein, or arabinoxylans. The increase in G' was not observed in pure starch samples. The decrease in G' of both doughs during holding at 20 °C may be explained by continuous mechanical stress to the samples.

Power−time curves were measured in starch suspensions and dough samples heated to simulate baking. The heat flow (μW/g of starch) was lower in rye starch and rye dough than in wheat starch and dough during the period of 2−48 h after baking. This might be related to different rates or intensities of phase transition processes (crystallization and glass transition) in rye and wheat dough after baking. Silverio and coauthors[27] suggested that the heat flow during the first 5−10 h is related to the amylose crystallization.

Wide-Angle X-ray Diffraction. Figure 3 provides wide angle X-ray diffractograms of rye, wheat, corn, and potato starches, maize amylopectin, and potato amylose. A high degree of similarity is observed between rye starch, wheat starch, and amylopectin isolated from high-amylopectin maize. Peaks at 15.3, 17, 18.5, and 23.1° (2θ) correspond to the A-type starch pattern[8,28] and are clearly present in the spectra of Figure 3. The native corn starch sample (40% amylose) has an XRD pattern similar to B-type starches characteristic of potato starch (not shown), having peaks around 17, 20, and 22−23° (2θ).[29,30]

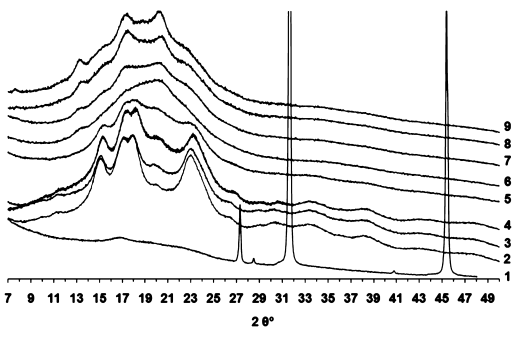

Figure 3. X-ray diffractograms of starch fractions, starches, flour, dough, and breads (1, amylose from potato; 2, amylopectin from maize; 3, rye starch; 4, rye flour; 5, rye dough; 6, rye bread, 0 h; 7, rye bread made from mix, sourdough, 11 days; 8, rye bread made from *L. plantarum*, 22 days; 9, rye bread made from *L. brevis*, 22 days).

The peaks characteristic to amylose preparation from potato had 2θ values of 27.4, 31.7, and 45.5° (Figure 3) and additionally 56.5, 66.3, and 75.3° (2θ) (not shown).

The A-type rye and wheat starch pattern became weaker after flour hydration during dough making and disappeared after baking (Figure 3). The XRD spectra of fresh rye and wheat breads (not shown) were almost identical and showed both the maximum at 20.1° and small peaks at 13.4°. The peaks at 13.4° and 20° (2θ) are reported to be typical for cocrystallized Va-type (anhydrated) amylose as single helices with alcohols or fatty acids.[31,32,3] Those structures may correspond to bright longish structures seen in Figure 1. With bread aging, the peaks around 15, 17, and 23° (2θ) reappear and a new peak appears at 7° (2θ) (Figure 3) in all bread samples studied. The typical A-starch double XRD pattern at 17 and 18° (2θ) in flour and dough changed after baking and storage into a single peak at 17° (2θ) for both wheat (not shown) and rye breads. The peaks at 15.0, 17.0, 22.2, and 24.0° (2θ) are characteristic of the B-type crystalline phase, which might include water in the crystalline structure.[23] Diffraction peaks that appear at 8, 13, and 20° (2θ) characterize a 6-fold single-helix structure (V6), whereas a 7-fold helical polymorph is characterized by peaks at 7, 12.5, and 18.5°, and the V8 structure is characterized by peaks at 17 and 22° (2θ).[33]

The crystallinity of wheat and rye flour, starch, dough, and bread was calculated from their diffractograms. The degree of crystallinity of starch in fresh rye bread (Table 1) increased from 3 to 21% in 11-day-old bread and in wheat bread increased from 3 to 26%. The degree of crystallinity of the bread starch fraction increased during rye bread staling up to 60% for rye bread and up to 80% for wheat bread. Using hetero- (*L. brevis*) or homofermentative species (*L. plantarum*) for sourdough fermentation did not affect the rye bread XRD pattern after storage (Figure 3).

Table 1. Degree of Crystallinity of Starch Fractions, Starch, and Bread Samples

sample	degree of crystallinity, %	
amylopectin from maize	25	
amylose from potato	41	
corn starch	25	
potato starch	29	
wheat starch	20	
rye starch	20	
	rye	wheat
flour	18	23
dough	14	20
bread, 0 h	3	3
bread, 5 h	9	9
bread, 11 days	21	26

Proton-Decoupled ^{13}C NMR and ^{31}P NMP Spectra of Starches. The chemical structure of rye and wheat starch was studied using ^{13}C and ^{31}P NMR. In linear amylose molecules only six peaks corresponding to the different glucose carbons appear in the proton-decoupled ^{13}C NMR spectra (Figure 4A). In the spectra of amylopectin, rye starch, and wheat starch we observe several smaller signals at all six carbon positions in addition to the C1−C6 signals of linear amylose and the linear part of amylopectin. For example, in addition to the main signal for the C4 glucose unit in rye starch, five smaller signals with comparable intensities are visible (Figure 4B). The peaks belong to the carbons of amylopectin in glucose units at the terminal ends, branching units, and units linked through (4→1)-α, (1→4)-α, (6→1)-α bonds to the branching glucose molecules. Using 2D methods (^{1}H−^{1}H COSY, HSQ, HMBC) these six signals are sorted out. These primed signals in Figure 4 belong to (1→4)-linked α-D-glucopyranosyl units with free 4-OH groups attached through C4 from branched amylopectin

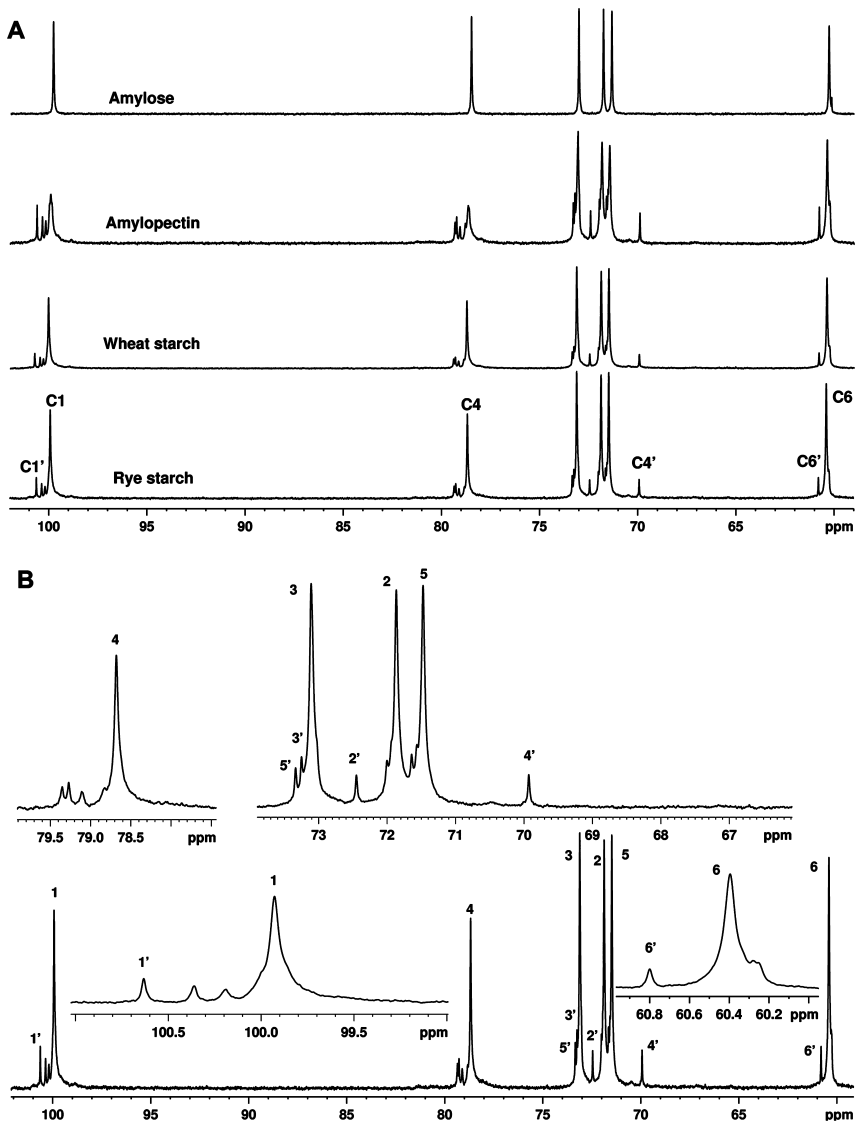

Figure 4. ^{13}C NMR spectra of amylose, amylopectin, and wheat and rye starches (A) and ^{1}H-decoupled 200 MHz ^{13}C NMR spectra of rye starch (B) in DMSO-d_6 solution at 313 K. Carbon signals from end glucose units of branches are primed.

end units. Exceptional positions a of the 4′ carbon signal at 70 ppm and a corresponding 4′ proton signal at 3.08 ppm (data not shown) are useful reference signals for the determination of degree of branching in starches. There are two different types of C6 carbons in starch glucose units: free side-chain CH_2OH groups and (1→6)-α-CH_2O-bridge groups. Despite this, ^{13}C signals of both types resonate within a narrow 0.4 ppm interval at 60 ppm. The only outstanding ^{13}C signal in this region is C6 from a terminal glucose unit with a free 4-OH group. Diastereotopic protons at this C6 have a comparatively strong chemical shift difference.

The ^{13}C NMR spectra of rye and wheat starch (Figure 4A) are very similar, and only some differences in the intensities of carbons corresponding to terminal glucose units (C′) compared those of the linear part (C) are observed. This can be caused by differences in amylose content and/or branching degree and chain lengths of amylopectin. By comparison of the signal intensity of primed carbons to all carbons of amylopectin, it can be calculated from the C1 signal that the percentage of terminal glucose units with a free 4-OH group in amylopectin was 10% and that in wheat and rye starches was 8%. The

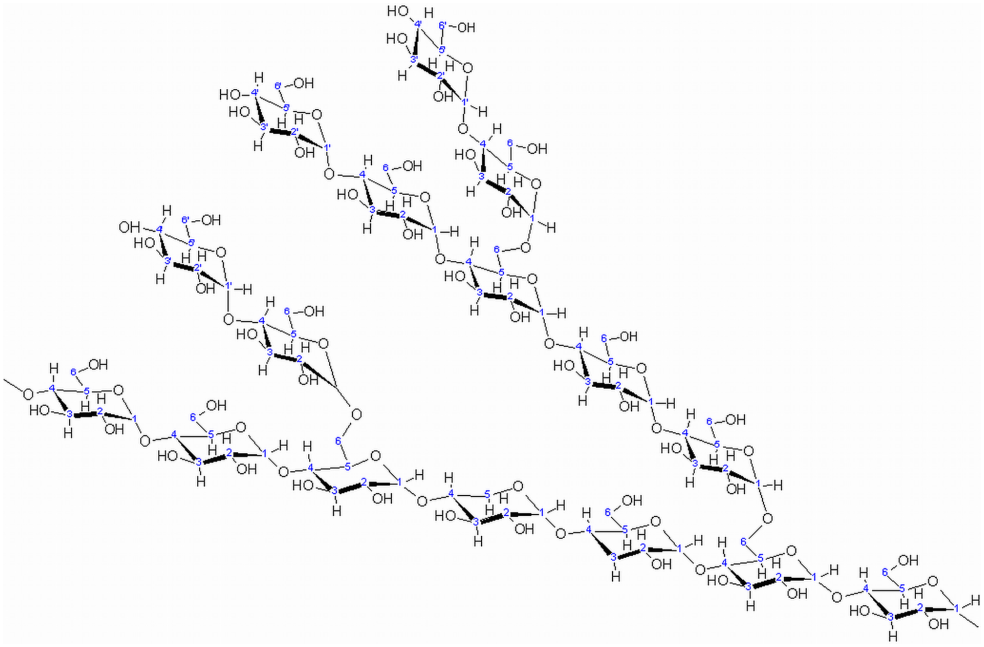

Figure 5. Schematic structure of amylopectin amorphous lamella region with ^{13}C and ^{1}H NMR signals. Primed numbers correspond to the carbon atoms of the end glucose units.

spectra suggest that, like amylose content, amylopectin structures (Figure 5) in rye and wheat starch are very similar. **^{31}P NMR spectra** of different starches are given in Figure 6. The significant difference observed between wheat and rye

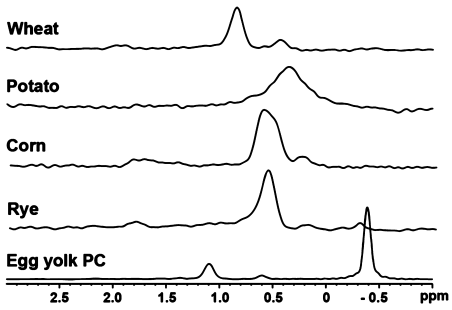

Figure 6. ^{31}P NMR spectra of starches (dissolved in DMSO-d_6). Egg yolk PC, egg yolk phosphatidylcholine (as reference).

starches can be caused by different compositions of the starch granule membrane, the phospholipids, and phosphate monoesters[34] or incorporated amylose−lipid complexes of rye and wheat starch.[11,35] Most of the cereal starch lipids in amylose−lipid complexes are lysophospholipids. Wheat starch contains 70% lysophosphatidylcholine, 20% lysophosphatidylethanolamine, and 10% lysophosphatidylglycerol.[36] Finnie and coauthors[37] reported that wheat starch contains phosphatidylcholine (70%), lysophosphatidylcholine (12%), phosphatidylethanolamine (8%), and phosphatidylglycerol (5%) located on the surface of wheat starch. Other phopholipids (lysophosphatidylglycerol, phosphatidylinositol) were reported to form 1−2% of the total phospholipid content in starch surface.

^{13}C Cross-Polarization Magic Angle Spinning Nuclear Magnetic Resonance (^{13}C CP MAS NMR). ^{13}C CP MAS NMR was used in parallel with XRD to study the changes in starch structure during baking. Although the lines of starch in solid phase ^{13}CP MAS NMR spectra (Figure 7B) are much broader than those dissolved in DMSO (Figure 4), significant changes in peak intensities corresponding to amylopectin carbons C1 (90−110 ppm) and C6 (63−60 ppm) during bread processing and storage are observed. Several changes in the C2, C3, and C5 (69−78 ppm) region, especially at 73.8−74.4 ppm, corresponding to the C3 and C4 (82 ppm) area are also observed.

Decomposition of the C1 and C6 resonances (Figure 7B) was carried out to better interpret differences and changes in the crystalline, semicrystalline, or amorphous structure of starch during rye bread baking and storage. The C1 region at 110−99 ppm was decomposed to five peaks (A−F), and the C6 region at 63−59 ppm was decomposed to three peaks (C6-H, C6-I, C6-J). peak A (103 ppm) in the C1 region is typical of the V-type single helix[38−41] with eight glucose cycles per turn[31] and the amorphous content (junction points of amylopectin double helices).[38−41] Peak A dominates in both the amylose and bread spectra. For amylopectin, the proportion of peak A was about 30% and that in starch and breads was 40−50%. The difference can be related to 22−25% amylose content in starch. In addition, rye and wheat starches and flour spectra displayed three peaks the in C-1 region: 101.5 (peak B), 100.5 (peak C), 99.5 (peak D) ppm are common to amylopectin (Figure 7) and correspond to three nonidentical sugar residues.[41] Peaks C and

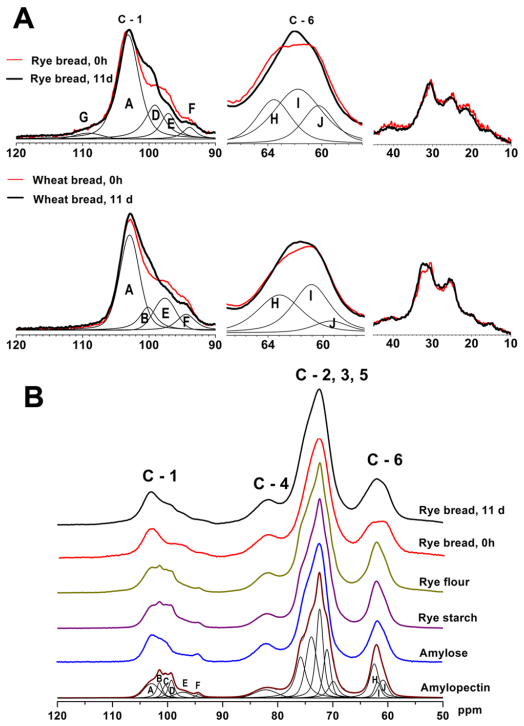

Figure 7. ^{13}C CP MAS NMR spectra of rye and wheat bread (A) and amylopectin with decomposition of resonances, amylose, rye starch, flour, and bread (B). Interpretations of the individual components: C1-A (102.9−103.2 ppm), amorphous region of amylopectin (branching points) and V-type single helix; C1-B (101.4−101.5 ppm), double helices; C1-C (100.1−100.5 ppm), double helices; C1-D (99.1−99.8 ppm), double helices; C1-E (96.4−97.7 ppm), glucose units near α-(1→6) linkages within the branched regions; C1-F (93.8−94.7 ppm), associated with constrained linkages; C1-G (106.9−109.1 ppm), C1 of rye cellulose.

D are characteristic of crystalline B-type double helices and correspond to two nonidentical sugar residues,[41] peak E can be related to the glucose units near α-(1→6) linkages within the branched regions, and peak F can be associated with constrained linkages.[11,31,38,41]

During baking of wheat and rye dough, the change in relative intensity of line A during baking and storage is not observed. Common to both of these breads during baking is an observed decrease in signal intensity in the region related to the crystalline structure (102−99 ppm, peaks B, C, and D) and an increase in relative intensity of the broad peak in the range of 98−96 ppm, which corresponds to a noncrystalline region. During staling, the area of this region decreased and that of the crystalline region (101−99 ppm) increased (Figure 7A). During staling of rye and wheat bread, the rye bread displayed a maximum at 99.3 ppm, whereas wheat bread displayed its maximum at 100.1 ppm. This suggests that these breads display differences in starch retrogradation.

NMR spectra showed changes during baking and storage also in the C6 region. Rye and wheat flour and starch all had a dominating peak at 62 ppm (Figure 7A). Upon baking, the maximum of the peak split and shifted from 62 to 61 ppm. During staling, the C6 peak became sharper again and obtained a maximum intensity at 62 ppm in both rye and wheat breads (Figure 7A). The effect can be explained by loss and reformulation of crystalline structure during baking and staling, respectively.

Additionally to the C1−C6 region, peaks at 173.6, 171.5, 160.6, 129.6, 106.1−109, 40.2, 31.5, 30.7, 25.1, and 21.6 ppm are observed in the MAS NMR spectra of bread (Figure 7A). The peak at 106.1−109 ppm (C1-G) is observed only in rye flour and bread, not in starch. The C1-G line recorded at 108 ppm in flour peaked at 109.1 ppm in rye bread and then shifted to 107 ppm during storage. This is probably explained by the presence of cellulose (1−3%)[42,43] or mixed-linkage (1→3)(1→4)-β-D-glucans (1.5−2.5%)[44,45] in dark rye flour.

DISCUSSION

The main fundamental questions are (1) what are the mechanisms causing the staling of bread and (2) what causes the differences in staling between wheat bread and rye sourdough bread. The recrystallization of starch is one of most important components affecting staling.[3,6]

Supposing that after gelatinization of starch during baking, the amylose and amylopectin start to crystallize in hydrated forms (for example, Vh or B-type) that contain about 30% water.[11] A considerable amount of water (15 g/100 g of bread) can be removed from the crumb amorphous phase into the crystalline structures from starch during bread storage. This reduction in water content in the amorphous bread structures can explain the dry mouthfeel in aged breads. According to XRD analysis an increase in the degree of crystallization occurs (Table 1). Taking into account a crystallinity change of 18% during staling, it can be calculated that about 3 g of water/100 g of bread can be bound into the crystalline structures of starch. That makes all together about 7.5% of water in bread, which might cause both the observed dry mouthfeel and an increase in bread firmness during storage.

The main question is whether the starch crystallizes out in hydrated forms in amounts sufficient to explain the staling. Comparison of X-ray spectra (Figure 3) as well as ^{13}C MAS NMR spectra (Figure 7) of flour and 11-day-stored bread revealed clear differences in the patterns of flour and bread. This can partly be explained by recrystallization of the starch in hydrated crystalline forms.[3,41] The XRD spectra of aged bread resembles more water-containing B-type potato than A-type wheat and rye starch spectra, suggesting that the B-type is preferred in aged bread. The development during storage of a B-type pattern in the crumb has been suggested also by Primo-Martin and coauthors.[3]

A water content >43% leads to the development of a B-type pattern, whereas a water content <29% leads to an A-type pattern.[46] The water content in rye bread was 42% and that in wheat bread, 39%. This difference in water content can partly explain the different patterns of C1 region in ^{13}C MAS spectra.

The decrease in water activity expected during crystallization in chemically bound forms is not being observed in this study. This might be explained by slow sensitivity of water activity to soluble compound concentration in the noncrystalline phase and parallel processes such as glass transition that could occur during staling. Incorporation of water from the bread crumb into the crystalline structures of starch during bread storage will increase the concentration of solubles in bread and can also initiate the transition from a rubber into a glassy state, which

might explain the increase in firmness and storage module during bread staling.[47]

The structural differences during starch retrogradation of rye and wheat bread are relatively small, probably because of the high similarity of the chemical structures of wheat and rye starch. Significant differences in the ^{13}C NMR spectra of rye and wheat starch dissolved in DMSO were not observed. Analyzing the well-resolved C-1 and C-4 areas in starch spectra, we observed five additional lines with almost equal intensity besides all of the major lines corresponding to the amylase type structure. We are able to designate only terminal glucose unit carbons; three others belong to carbons linked directly to the branching glucose unit of amylopectin, and one belongs to the branching unit itself.

As the percentage of branching glucose units is calculated from ^{13}C NMR spectra of amylopectin is 10%, the branching region in amylopectin takes $5 \times 10 = 50\%$ of the glucose units. Taking into account that the average chain lengths of rye and wheat amylopectin are 20−22 and 23, respectively,[25] about 2.5 medium glucose cycle chains per 10 branching glucose cycles can be calculated according to the structure postulated in Figure 5.

Overall, the results show that regardless of the type of rye and wheat, the A-type starch chemical structure is very similar, and they transform after baking into hydrated crystalline forms during storage. However, some differences in the staling process were observed. According to ^{13}C NMR the major crystallites that formed during staling were different and the relative crystallinity of starch in rye sourdough bread is less and increases more slowly than in wheat bread. This can be related to differences in starch structures (phospholipids, granule size), protein matrix, content of water, and pH, as well as the scalding of flour.

AUTHOR INFORMATION

Corresponding Author

*Phone: +372 5221490. Fax: +372 6408282. E-mail: anna@tftak.eu.

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Notes

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REFERENCES

(1) Lund, D.; Lorenz, K. J. Influence of time, temperature, moisture, ingredients, and processing conditions on starch gelatinization. *Crit. Rev. Food Sci. Nutr.* **1984**, *20*, 249−273.

(2) Belitz, H.-D.; Grosch, W.; Schieberle, P. Starch. Structure and properties of starch granules. In *Food Chemistry*, 4th ed.; Belitz, H.-D., Grosch, W., Schieberle, P., Eds.; Springer-Verlag: Berlin, Germany, 2009.

(3) Primo-Martin, C.; van Nieuwenhuijzen, N. H.; Hamer, R. J.; van Vliet, T. Crystallinity changes in wheat starch during the bread-making process: starch crystallinity in the bread crust. *J. Cereal Sci.* **2007**, *45*, 219−226.

(4) Czuchajowska, Z.; Pomeranz, Y. Different scanning calorimetry, water activity, and moisture contents in crumb center and near-crust zones of bread during storage. *Cereal Chem.* **1989**, *66*, 305−309.

(5) Piazza, L.; Masi, P. Moisture redistribution throughout the bread loaf during staling and its effect on mechanical properties. *Cereal Chem.* **1995**, *72*, 320−325.

(6) Ask, L.; Nair, B.; Asp, N.-G. Effect of scalding procedures on the degradation of starch in rye products. *J. Cereal Sci.* **1991**, *13*, 15−26.

(7) Imberty, A.; Buleon, A.; Tran, V.; Perez, S. Recent advances in knowledge of starch structure. *Starch/Staerke* **1991**, *43*, 375−384.

(8) Maningat, C. C.; Seib, P. A.; Bassi, S. D.; Woo, K. S.; Lasater, G. D. Wheat starch: production, properties, modification and uses. In *Starch: Chemistry and Technology*, 3rd ed.; BeMiller, J., Whistler, R., Eds.; Academic Press: New York, 2009; pp 441−510.

(9) Wang, T. L.; Bogracheva, T. Y.; Hedley, C. L. Starch: as simple as A, B, C? *J. Exp. Bot.* **1998**, *49*, 481−502.

(10) Blazek, J.; Gilbert, E. P. Application of small angle X ray and neutron scattering techniques to the characterization of starch structure: a review. *Carbohydr. Polym.* **2011**, *85*, 281−293.

(11) Perez, S.; Baldwin, P. M.; Gallant, D. J. Structural features of starch granules I. In *Starch: Chemistry and Technology*, 3rd ed.; BeMiller, J., Whistler, R., Eds.; Academic Press: New York, 2009; pp 149−192.

(12) Imberty, A.; Chanzy, H.; Perez, S.; Buleon, A.; Tran, V. The double-helical nature of the crystalline part of A-starch. *J. Mol. Biol.* **1988**, *201*, 365−378.

(13) Popov, D.; Burghammer, M.; Buleon, A.; Montesanti, N.; Putaux, J. L.; Riekel, C. A-amylose single crystalls: unit cell refinement from synchrotron radiation microdiffraction data. *Macromolecules* **2006**, *39*, 3704−3706.

(14) Eliasson, A.-C.; Larsson, K. *Cereals in Breadmaking − A Molecular Colloidal Approach*; Marcel Dekker: New York, 1993.

(15) Koehler, P.; Wieser, H. Comparative studies of high Mr subunits of rye and wheat. III. Localisation of cysteine residues. *J. Cereal Sci.* **2000**, *32*, 189−197.

(16) Autio, K. Functional aspects of cereal cell-wall polysaccharides. In *Carbohydrates in Food*, 2nd ed.; Eliasson, A.-C., Ed.; CRC Press: New York, 2006; pp 168−207.

(17) Buksa, K.; Nowotna, A.; Praznik, W.; Gambus, H.; Ziobro, R.; Krawontka, J. The role of pentosans and starch in baking of wholemeal rye bread. *Food Res. Int.* **2010**, *43*, 2045−2051.

(18) Hammes, W. P.; Ganzle, M. G. Sourdough breads and related products. In *Microbiology of Fermented Foods*, 2nd ed.; Woods, B. J. B., Ed.; Blackie Academic/Professional: London, U.K., 1998; Vol. *1*, pp 199−216.

(19) Arendt, E. K.; Ryan, L. A. M.; Dal Bello, F. Impact of sourdough on the texture of bread. *Food Microbiol.* **2007**, *24*, 165−174.

(20) Siljeström, M.; Björck, I.; Eliasson, A.-C.; Lönner, C.; Nyman, M.; Asp, N. G.. Effects on polysaccharides during baking and storage of bread—in vitro and in vivo studies. *Cereal Chem.* **1988**, *65*, 1−8.

(21) Verwimp, T.; Vandeputte, G. E.; Marrant, K.; Delcour, J. A. Isolation and characterisation of rye starch. *J. Cereal Sci.* **2004**, *39*, 85−90.

(22) Mihhalevski, A.; Sarand, I.; Viiard, E.; Salumets, A.; Paalme, T. Growth characterization of individual rye sourdough bacteria by isothermal microcalorimetry. *J. Appl. Microbiol.* **2011**, *110*, 529−540.

(23) Ribotta, P. D.; Cuffini, S.; Leon, A. E.; Anon, M. C. The staling of bread: an X-ray diffraction study. *Eur. Food Res. Technol.* **2004**, *218*, 219−223.

(24) Morcombe, C. R.; Zilm, K. W. Chemical shift referencing in MAS solid state NMR. *J. Magn. Reson.* **2003**, *162*, 479−486.

(25) Gomand, S. V.; Vervimp, T.; Goesaert, H.; Delcour, J. A. Structural and physicochemical characterization of rye starch. *Carbohydr. Res.* **2011**, *346*, 2727−2735.

(26) Hug-Iten, S.; Handschin, S.; Conde-Petit, B.; Escher, F. Changes in starch microstructure on baking and staling of wheat bread. *LWT−Food Sci. Technol.* **1999**, *5*, 255−260.

(27) Silverio, J.; Svensson, E.; Eliasson, A.-C.; Olofsson, G. Isothermal microcalorimetric studies on starch retrogradation. *J. Therm. Anal. Calorim.* **1996**, *47*, 1179−1200.
(28) Xie, F.; Liu, H.; Chen, P.; Xue, T.; Chen, L.; Yu, L.; Corrigans, P. Starch gelatinization under shearless and shear conditions. *Int. J. Food Eng.* **2006**, *2*, article 6.
(29) Kuakpetoon, D.; Wang, Y.-J. Structural characteristics and physicochemical properties of oxidized corn starches varying in amylose content. *Carbohydr. Res.* **2006**, *341*, 1896−1915.
(30) Frost, K.; Kaminski, D.; Kirwan, G.; Lascaris, E.; Shanks, R. Crystallinity and structure of starch using wide angle X-ray scattering. *Carbohydr. Polym.* **2009**, *78*, 543−548.
(31) Paris, M.; Bizot, H.; Emery, J.; Buzare, J. Y.; Buleon, A. NMR local range investigations in amorphous starchy substrates I. Structural heterogeneity probed by ^{13}C CP−MAS NMR. *Int. J. Biol. Macromol.* **2001**, *29*, 127−136.
(32) Le Bail., P.; Bizot, H.; Ollivon, M.; Keller, G.; Bourgaux, C.; Buleon, A. Monitoring the crystallization of amylose-lipid complexes during maize starch melting by synchrotron X-ray diffraction. *Biopolymers* **1999**, *50*, 99−110.
(33) Gidley, M. J.; Bociek, S. M. Molecular organization in starches: a ^{13}C CP/MAS NMR study. *J. Am. Chem. Soc.* **1985**, *107*, 7040−7044.
(34) Kasemsuwan, T.; Jane, J.-L. Quantitative method for the survey of starch phosphate derivatives and starch phospholipids by ^{31}P nuclear magnetic resonance spectroscopy. *Cereal Chem.* **1996**, *73* (6), 702−707.
(35) Jane, J.-L. Structural features of starch granules II. In *Starch: Chemistry and Technology*, 3th ed.; BeMiller, J., Whistler, R., Eds.; Academic Press: New York, 2009; pp 193−236.
(36) Morrison, W. R. Lipids in cereal starches: a review. *J. Cereal Sci.* **1989**, *8*, 1−15.
(37) Finnie, S. M.; Jeannotte, R.; Morris, C. F.; Giroux, M. J.; Faubion, J. M. Variation in polar lipids located on the surface of wheat starch. *J. Cereal Sci.* **2010**, *51*, 73−80.
(38) Therien-Aubin, H.; Janvier, F.; Baille, W. E.; Zhu, X. X.; Marchessault, R. H. Study of hydration of cross-linked high amylose starch by solid state ^{13}C NMR spectroscopy. *Carbohydr. Res.* **2007**, *342*, 1525−1529.
(39) Therien-Aubin, H.; Zhu, X. X. NMR spectroscopy and imaging studies of pharmaceutical tablets made of starch. *Carbohydr. Polym.* **2009**, *75*, 369−379.
(40) Tang, H. R.; Hills, B. P. Use of ^{13}C MAS NMR to study domain structure and dynamics of polysaccharides in the native starch granules. *Biomacromolecules* **2003**, *4*, 1269−1276.
(41) Baik, M. Y.; Dickinson, L. C.; Chinachoti, P. Solid-state ^{13}C CP/MAS NMR studies on aging of starch in white bread. *J. Agric. Food Chem.* **2008**, *51*, 1242−1248.
(42) Zhang, J.; Zhang, J.; Lin, L.; Chen, T.; Zhang, J.; Liu, S.; Li, Z.; Ouyang, P. Dissolution of microcrystalline cellulose in phosphoric acid—molecular changes and kinetics. *Molecules* **2009**, *14*, 5027−5041.
(43) Liukkonen, K.-H.; Heiniö, R.-L.; Salmenkalloo-Marttila, M.; Autio, K.; Katina, K.; Poutanen, K. Rye. In *Bakery Products: Science and Technology*; Hui, Y. H., Ed.; Blackwell Publishing: Ames, IA, 2006; pp 109−122.
(44) Henry, R. J. Pentosan and (1→3),(1→4)-β-glucan concentrations in endosperm and wholegrain of wheat, barley, oats and rye. *J. Cereal Sci.* **1987**, *6*, 253−258.
(45) Roubroeks, J. P.; Andersson, R.; Aman, P. Structural features of (1→3),(1→4)-β-D-glucan and arabinoxylan fractions isolated from rye bran. *Carbohydr. Polym.* **2000**, *42*, 3−11.
(46) Osella, C. A.; Sanchez, H. D.; Carrara, C. R.; de la Torre, M. A.; Buera, M. P. Water redistribution and structural changes of starch during storage of a gluten-free bread. *Starch/Staerke* **2005**, *57*, 208−216.
(47) Cesaro, A.; Sussich, F. Plasticization: the softening of materials. In *Bread Staling*; Chinachoti, P., Yael Vodovotz, Y., Eds.; CRC Press, Boca Raton, FL, 2001; Chapter 2.

PUBLICATION VII

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Quality of triticale cultivars suitable for growing and bread-making in northern conditions

M. Tohver [a,*], A. Kann [b], R. Täht [b,*], A. Mihhalevski [b], J. Hakman [b]

[a] *Institute of Experimental Biology, Estonian Agricultural University, Harku, EE 76902 Harjumaa, Estonia*
[b] *Tallinn Technical University, Ehitajate tee 5, 12618 Tallinn, Estonia*

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Abstract

Rye and wheat are the major cereals consumed by people of northern and eastern Europe. However, it has become important to widen the use of cereal products. Attempts have been done to adapt western triticale cultivars to northern growing conditions. Serious investigations of triticale bread commenced as late as in 1997 in Estonia. The first attempts to grow the triticale cultivars Modus, Dato, Presto, SV 92280 in Estonian conditions have been successful. They are productive, resistant to leaf and stem rust, powdery mildew, and with good grain quality.

The aim of this work was to investigate the bread-making quality of western winter triticale cultivars grown in Estonia. The following grain quality parameters were estimated: grain protein content, SDS-sedimentation, falling number, water absorption, bread loaf volume.

HMW glutenin subunit composition, which was used to predict the baking quality, was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Seven different alleles were revealed in the set of 12 triticale cultivars. The best cultivars had HMW glutenin subunits 2* encoded by the *Glu-A1* locus, 7+26 and 7+19 encoded by the *Glu-B1* locus.

Keywords: Hexaploid triticale; Grain and bread-making quality; SDS–PAGE; HMW glutenin subunits

1. Introduction

The hexaploid triticale (×*Triticosecale* Wittmack) (AABBRR) created by crossing species of wheat (*Triticum*) (AABB) and rye (*Secale*) (RR) combines the properties of both parental cereals. Triticale has the high yield potential and grain quality of wheat and the resistance to pathogens of rye. When compared to wheats, modern hexaploid winter triticale cultivars show higher yields and good adaptation to northern environments. Their flour is rich in proteins (average 14–15%), suggesting a promising use for the production of human foods (Täht et al., 1998; Varughese, Pfeiffer, & Peña, 1996).

The good growing areas for triticale are Middle and South Europe – Germany, the Czech Republic, Poland, Austria, Portugal (Igrejas, Guedes-Pinto, Carnide, & Branlard, 1999; Lafferty & Lelley, 2001; Šašek et al., 1988; Weipert, 1996; Wolski, 1986), USA (Varughese et al., 1996), Canada (Bushuk & Larter, 1980), Australia (Cooper, 1986), and Greece (Doxastakis, Zafiriadis, Irakli, Marlani, & Tananaki, 2002).

Estonia belongs to northern countries where it is still possible to cultivate rye, wheat, and triticale. The problems are caused by Estonian climate (cool, moist, rainy) – crops have unstable yields and low quality. Systematic investigation of triticale as a perspective food-fodder and technical culture was initiated in Estonia by the Estonian Agricultural University and Tallinn Technical University in 1995 (Laur, Jaama, Kasearu, & Vooremäe, 1997). In 1996 and 1997 a field trial with cultivars originated from Germany and Sweden was carried out at the Jõgeva Plant Breeding Institute with the cultivars Modus, SV 92280, Dato and Vision, and 9 breeding lines (Tupits & Kukk, 1999). The trials showed that the cultivars of triticale had yielding capacity exceeding that of wheat and rye ones,

* Corresponding authors.
E-mail addresses: maimu.tohver@mail.ee (M. Tohver), Kriss38@hot.ee (R. Täht).

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and medium winter hardness in Estonian climatic conditions.

The quality of kernel is a complex of physical and chemical characteristics whose expression depends on their genetic nature and influence of environment (Johansson, 2002; Johansson & Svensson, 1998). Methods of predicting genetically better cultivars and breeding lines are of great importance. The investigation of storage proteins of cereals by methods of electrophoresis is a useful tool for these purposes. Various allelic variants of the genes for the high-molecular-weight subunits of glutenin are particularly important for determining wheat gluten and dough elasticity (Payne, 1987; Shewry, Popineau, Lafiandra, & Belton, 2001) using for these purposes only half a kernel and being as predictors of grain quality. The verified correlations between bread-making quality and occurrence of specific HMW subunits of glutenin have been taken advantage of by wheat breeders, using SDS–PAGE of proteins as screening test for bread-making quality (Johansson, Svensson, & Heneen, 1995). The variation in high-molecular-weight glutenin subunit composition has been reported to account for up to 70% of the variation in breadmaking quality of European wheats (Branlard & Dardevet, 1985).

In hexaploid wheat (*Triticum aestivum* L.) the HMW glutenin subunits are encoded by genes located on chromosomes 1A, 1B, and 1D, in hexaploid triticale - on chromosomes 1A, 1B, and 2R. In the investigation of the *Glu-1* alleles (Brzezinski & Lukaszewski, 1998) it was shown that the gene pool of hexaploid winter triticale contains relatively high proportion of the *Glu-1* alleles, which correlates well with good bread-making quality.

During the mixing of wheat or other cereal flour with water to make dough, proteins form gluten that is the basis of dough functionality. Gluten can be divided into two groups – gliadins and glutenins, which together confer the properties of elasticity (strength) and extensibility (viscosity) (Kasarda, 1989). These unique properties of wheat gluten are the basis of the wide range of wheat-derived food products. Since the wheat parent of hexaploid triticale originates from tetraploid wheat and does not contain the D-genome, it also lacks the bread-making quality of hexaploid wheat (Lafferty & Lelley, 2001).

Because of influence of pentosans and α-amylase, the baking quality of rye is lower than that of wheat. As the genome of triticale contains the chromosomes of rye, the secalins encoded by rye chromosomes have a noticeable influence on the bread quality of triticale. The presence of the 1RS segment in triticale is responsible for significant reduction in rheological properties and overall gluten strength, and significant increase in dough stickiness (Peña & Amaya, 1992).

The contribution of the seed storage protein groups to dough properties of wheat flour has been studied extensively (Branlard & Dardevet, 1985; Branlard, Dardevet, Saccomano, Lagoutte, & Gourdon, 2001; Gupta & Mac Ritchie, 1994; Hamer, Weegels, & Marseille, 1992; Payne, 1987; Sontag-Strohm & Juuti, 1997; Uhlen, 1990). The functional properties of triticale proteins, such as water absorption, viscosity, gelation, which influenced by agronomic factors, and storage, composition and processing of bread have been neglected (Dervas, Doxastakis, Hadjisavva-Zinoviadi, & Triantafillakos, 1999).

The aim of the present study was: (1) to find the most suitable triticale cultivars to be used in local brown and white bread industry; (2) to study the influence of the alleles at *Glu-1* loci on bread-making quality.

2. Materials and methods

2.1. Materials

Seed samples of 12 hexaploid triticale cultivars were obtained from the Jõgeva Plant Breeding Institute, Viljandi Control Centre of Plant Production (Estonia), and Svalöf (Sweden). Among them the cultivars Lasko, Prego, Pinokio, Dagro, Tewo, Presto are originated from Poland, SW 98578 and SV 92280 – from Sweden, Dato, Vision, Modus – from Germany, Moreno – from the USA.

The cultivars were grown in Estonia since 1995, and the biochemical and baking investigations were performed in 1999–2001. The experiments were carried out at Tallinn Technical University (gluten content, baking), at the Institute of Experimental Biology of the Estonian Agricultural University (HMW glutenin subunit composition), and at Estonian Control Centre of Plant Production (protein and moisture content, falling number, Zeleny sedimentation test, water absorption).

2.2. Chemical analyses

Grains were milled in PERTEN instruments mill 3170 and QC-109 Labhim mill to pass a 0.8 mm screen.

Nitrogen content was determined by using Kjeltec 1015 digester and Kjeltec Auto 1030 Analyser on wholemeal flour and is presented on a dry weight basis (Tecator Application Note, 1987). Nitrogen content was multiplied by a factor of 5.7 to determine protein content in wheat and triticale and 6.25 (ICC Draft Standard No. 167) to determine protein content in rye. All the determinations were expressed on a dry weight basis. Moisture content was determined by drying the samples at 105 °C to constant weight.

Crude gluten in the flour was determined by ICC standard No. 137/1982 using a Glutomatic 2200 instrument on white flour milled on a Brabender Quadromat Junior which gave extraction rates of 55–65%. A dough was prepared from a flour sample by adding a buffered 2% sodium chloride solution. Gluten washing was carried out

by Glutomatic 2200 using a solution of 2% sodium chloride. The residual water adherent to the gluten was removed by centrifugation and the reminder weighed.

2.3. Zeleny sedimentation test

For Zeleny sedimentation test the grains were milled on a special Brabender OHG Duisburg mill which gave extraction rates of 8–10%. The test was performed according to the procedure of EVS 765:2000 (the standard of the Estonian Republic; ICC, 1972, 1982). 3.2 g flour was weighed and placed in a 100 ml cylinder, and mixed for 5 min. 25 ml of test solution (180 ml of lactic acid and 200 ml isopropanol) was added to obtain a final concentration of the solution 3.5% of lactic acid and 17.5% of isopropanol. The cylinder was shaked for 10 min, and 5 min later the volume of the sediment was measured.

2.4. Falling number

Falling number which represents the activity of α-amylase was estimated with Falling Number 1800 according to the procedure of EVS 654:1999 (the standard of the Estonian Republic; ICC Standard No. 107/1). The method is based on the rapid gelatinisation of a suspension of flour in a boiling water bath and the subsequent measurement of the liquefaction of starch by alpha amylase present in the sample.

The grains were milled with a special PERTEN instruments mill for falling number. The flour and water suspension (7 ± 0.05 g flour and 25 ml water) was placed in viscometer tube into a boiling water bath. The time in seconds needed by the stirrer of viscometer to drop down is falling number.

2.5. Electrophoresis

Total proteins were extracted from individually ground grains in a mortar with a pestle until a fine powder was obtained. Reduced protein extracts were obtained by incubating the samples in SDS–Tris–HCl buffer pH 6.8 containing 0.125 M Tris, 2.75% sodium dodecyl sulphate (SDS), 10% (v/v) glycerol, 1% (w/v) DTT, and 0.005% bromophenol blue for 1 h at 70 °C. After centrifugation (14,000 rpm, 10 min), 20 μl of supernatant was used for electrophoresis. HMW glutenin subunits were separated in the presence of SDS at pH 8.3 for 22 h at 10 mA on 10% polyacrylamide gel. The running buffer contained 0.2 M glycine, 0.05 M Tris pH 8.3 and 0.1% SDS. After electrophoresis the gels were stained overnight with 0.05% Coomassie Brilliant Blue R-250 in water–methanol–glacial acetic acid (53:40:7) and destained in the solution of the same components (68:25:7), and photographed (D'Ovidio, Lafiandra, & Porceddu, 1996; Tohver, Täht, Kann, & Rahnu, 2000; Tohver, Kann, Täht, & Mihhalevski, 2001). The designation of triticale bands was performed on the basis of wheat genome, and HMW glutenin subunit bands were analysed according to the nomenclature of Payne and Lawrence (1983), UPOV (1994), and Brzezinski and Lukaszewski (1998).

2.6. Baking test

The baking formula was a typical wheat bread formula, but the wheat whole- grain flour was mixed with 10–70% triticale wholegrain flour (Table 1). Emulsifiers DRIV (E 471, E 260, E 262), Lecimax (E 472, E 300) and Balt (E 263, E 300) were also included in the bread formulas. The mix used was composed of non-fat milk powder, margarine, sugar, salt and baking yeast. Baking was performed using an automatic Panasonic™ baker's oven (Täht et al., 1998).

Spring wheat cultivar Heta was used in baking experiments (protein, 13.2%; FN, 376 s; Wac, 83.0%). Triticale flour was substituted for wheat flour up to 70% (Table 4). Flour (or flour blends) were stirred for 1 min in the bowl before adding the other ingredients (salt and yeast). Yeast was activated by warm water and sugar. The dough was mixed up to homogeneous consistency and then put into a fermentation cabinet at 31 ± 1 °C for 175 min at a relative humidity of 85%. The dough was scaled at 550 g, moulded, placed in a baking pan and returned to the fermentation cabinet, and proofed for 35 min at 35 ± 1 °C. After proofing the doughs of pan and round bread were baked in the oven for 40 min at 230 °C and for 30 min at 250 °C, respectively. The amount of water to be used was determined by the absorption value. Water absorption (Wac) was determined by centrifugation method (AOAC, 1984).

2.7. Bread volume

The loaf volumes were measured after baking using the rapeseed displacement method (AACC, 1983).

2.8. Statistical analysis

Statistical calculations (correlation coefficients and SD) were made using of MS Excel statistical package.

Table 1
Recipe of experimental breads (30% of triticale in wheat-triticale breads)

Ingredient	Amount (g)
Wheat flour	210
Triticale flour	90
Sugar	15
Milk powder	12
Margarin	10
Salt	5
Yeast	9
Water	210

3. Results and discussion

3.1. Grain protein and gluten content

Data describing the properties of triticale flour, dough, and the allelic variants of HMW gluten subunits for 13 cultivars are presented in Tables 2 and 3.

Protein content of flour is extremely important because almost all flour properties (gluten content, water absorption, mixing requirement, loaf volume) are highly correlated with protein content (Pomeranz, 1985). In 1999–2001 the protein content in the investigated cultivars varied from 9.7–14.5% with the average of 11.8% and with SD of 1.44. Protein content was higher in genotypes with HMW glutenin subunits 2* coded by the locus *Glu-A1,* and 7+19 and 7+26 coded by the *Glu-B1* (Table 3). Influence of environmental factors is demonstrated on the examples of the cultivar Tewo (Table 2). Tewo 1 and Tewo 2 are the same cultivar, grown up in a different environment. Tewo 2 had a higher protein content and falling number, lower bread volume and Zeleny number. In Estonia where the summer climate is variable, the lower protein content and falling number for Tewo 1 could be related with the different weather conditions in northern and southern Estonia. Tewo 1 was grown in southern and Tewo 2 in northern Estonia. Northern Estonia differs from southern area in the average of summer temperatures and in amount of rainfalls.

Gluten content was generally low, and in some cases was not detectable. The absence of gluten shows that triticale protein behaves as that of rye, and is too low to yield good quality bread. Correlation between protein content and volume of bread was positively significant

Table 2
The properties of dough and gluten of triticale cultivars

Cultivar	*Glu-A1*	*Glu-B1*	Gluten (%)	GPC (%)	FN (s)	VB (%)	Zeleny (ml)	Wac (%)
Lasko	2*	7+19	0	12.8	240	96.8	–	–
Prego	N	7+8	0	11.2	357	73.0	–	–
Pinokio	2*	13+16	4.2	10.4	200	73.0	–	–
Dagro	N	7+19	0	9.7	62	75.8	–	–
Presto	2*	7+26	26.5	13.7	180	97.0	17.3	55.5
Tewo 1	2*	7+19	0	12.6	88	109.8	21.0	56.1
Tewo 2	2*	7+19	0	14.5	195	94.4	17.3	65.0
Vision	2*	7+19	0	11.6	89	–	10.5	90.3
Dato	2*	7+19	19.0	12.5	128	90.1	17.0	90.3
	N	6+8						
Modus	N	7+26	16.0	11.0	–	88.3	–	87.5
SW 98578	N	13+16	0	9.7	62	75.8	–	–
SV 92280	2*	6+8	8.0	12.3	182	88.6	–	87.5
Moreno	2*	6+8	0	11.8	240	104.2	21.0	61.9
		7+26						
Average of columns				11.83	168.58	88.9	17.35	74.26
SD				1.44	87.85	12.36	3.84	15.97

GPC, grain protein content; FN, falling number; VB, volume of riticale bread with 30% of wheat flour – % from control wheat bread; Wac, water absorption; N, null allele; –, not determined; 0, not detectable. The data are the averages of three repetitions.

Table 3
Dough and gluten properties of allelic variants of triticale

Genotype		*n*	GPC (%)	Wac (%)	Zeleny (ml)	FN (s)	VB (%)
Glu-A1	*Glu-B1*						
2*	6+8	2	12.1	74.7	21.0	211	96.4
2*	7+19	5	12.8	75.4	16.4	149	97.8
2*	7+26	1	13.7	55.5	17.3	180	97.0
2*	13+16	1	10.4	–	–	131	74.4
N	13+16	1	9.7	–	–	62	75.8
N	7+8	1	11.2	–	–	357	73.0
N	7+19	1	9.7	–	–	62	75.8
N	7+26	1	11.0	87.5	–	–	88.3
Average			11.37	73.25	18.23	164.57	84.81
SD			1.45	13.22	2.43	101.52	11.17

GPC, grain protein content; Wac, water absorption; FN, falling number; VB, volume of bread – % from control wheat bread; *n*, number of cultivars.

($r = +0.696 \pm 0.228$), significantly negative between water absorption ($r = -0.559 \pm 0.339$), positive, but not significant between Zeleny number ($r = 0.177 \pm 0.492$). The rye proteins are not able to form gluten because of the structure of proteins, high content of pentosans, and high activity of alpha amylase (Weipert, 1996). In the cases of HMW glutenin subunits 2*, 7+19 and 7+26, gluten content was somewhat higher.

3.2. Falling number

The falling number influences the dough characteristics and bread properties, and shows also the preharvest sprouting. Preharvest sprouting is considered to be the main argument against using triticale for bread-making in regions with a moist climate. Investigating a large number of triticale cultivars enables to select the cultivars with satisfying quality. The viscoelastic properties of dough, as well as its ability to hold gas during fermentation and maintain the loaf shape, are found to be affected by the values of falling number. An extensive starch degradation prevents the formation of a sticky dough (Lafferty & Lelley, 2001). Flour with the optimum falling number is required to produce an optimum dough and optimum bread. Quite satisfactory baking performance showed flours with falling numbers 220–250 s, but poor results were obtained using flours with falling numbers below 120 s (Ingver & Koppel, 1998; Veskus & Kann, 1997). The falling numbers of investigated triticale cultivars varied from very low (62 s for SW 98578 and Dagro) and accordingly low volume of bread (75.8% from control wheat bread) to too high (357 s for Prego). Triticale flour with low falling number gives a soft dough which may be too fluid to bake into bread. Flour with high (more than 300 s) falling number yields a relatively stable dough, but the bread structure will be dense and its consistency hard. Volume of bread was satisfying in the case of cultivars Lasko, Presto, Tewo1 and Moreno (falling numbers 245, 180, 109.8 and 240, respectively). Although the falling number is considered as a varietal property, weather conditions have a marked effect on this grain quality characteristic (Tewo 1 and Tewo 2, Table 2).

3.3. Zeleny number

The Zeleny sedimentation value (SDS-sedimentation) was determined as one of the parameters for bread-making quality. The SDS-sedimentation values of the tested triticale cultivars varied from 10.5 to 21.0 ml, and were comparable to data for triticale cultivars found by Brzezinski and Lukaszewski (1998). These values are too low for good bread-making quality. The cultivars differing in *Glu-B1* alleles showed a slightly, though not significantly, higher sedimentation value in the presence of subunits 7+19 and 7+26. In these cases the volume of bread was highest. Earlier it was shown that subunits 1 and 2* encoded by chromosome 1A, and 6+8, 7+8 and 7+9 encoded by chromosome 1B do not correlate with strong gluten (Payne, Nightingale, Krattinger, & Holt, 1987; Uhlen, 1990). Other authors (Johansson et al., 1995) demonstrated that subunit 2* had a fairly good quality score showing a slight positive correlation with the Zeleny sedimentation volume. This agrees with the findings of the present work. Correlation between Zeleny number and volume of bread was significantly positive ($r = +0.931 \pm 0.216$), negative correlation was estimated between Zeleny number and water absorption ($r = -0.723 \pm 0.346$). As the falling and Zeleny numbers were low, the triticale breads were baked with an addition of wheat flour.

3.4. Baking

The results of the baking test are presented in Tables 2 and 4. Water absorption and dough consistency are important in relation to mixing, dividing, panning and proofing steps (Kasearu et al., 1997). For optimum development the dough must attain a balance between elastic and viscous properties. The dough should be neither too elastic nor too extensible. Water absorption is considered to be good if it remains in the range of 55–65% (Kasearu et al., 1997). In this investigation water absorption level was negatively correlated with loaf volume ($r = -0.831 \pm 0.249$) and protein content ($r = -0.559 \pm 0.339$). Table 2 demonstrates that Wac of the cultivars Vision, Dato, Modus and SV 92280 are too high for good bread. The loaf volume which is generally regarded as an important quality criterion is biggest for bread baked from flour of 12.6% protein content, of 21 ml Zeleny number, falling number of 88 s and Wac of 56.1%. The flour from the cultivar Moreno gave a good volume of bread with protein content of 12.7%, Zeleny number of 21 ml, falling number of 240 and Wac of 61.9%. It is in good accordance with the results concerning water absorption and protein content, however, falling number 88 s was too low for good bread. Therefore, experiments were carried out to bake breads with wheat additions. Loaf volumes of wheat breads prepared with triticale additions of 30%, 50% and 70% were comparable with that of 100% wheat bread (Table 4).

Table 4
Volume of bread (% from control wheat bread)

	% of triticale flour		
Cultivar	30%	50%	70%
Tewo1	109.8	104.9	101.4
Tewo 2	94.4	97.2	88.7
Moreno	104.2	99.3	102.8
Presto	97.9	94.4	91.6
Average	96.13	98.95	96.13
SD	6.81	4.44	7.02

This agrees with the data reported by Doxastakis et al. (2002), and Peña and Amaya (1992), that the triticale flour blends (up to 50%) with wheat flours produce breads with quality similar to that of breads made from wheat flours only. Sensory evaluation studies indicate that triticale can be used satisfactorily as a good ingredient in bread-making. All the loaves baked from flour of different cultivars of triticale mixed with wheat flour proved to be of acceptable quality and fit for human consumption. All the loaves had a pleasant yellow colour, a fresh and pleasant smell and taste (Tohver et al., 2000; Täht et al., 1998).

3.5. HMW glutenin subunits and baking quality

It is now well established that the glutenin fraction is the most important fraction related to bread-making quality (Branlard et al., 2001; Mac Ritchie, Du Cross, & Wrigley, 1990; Shewry et al., 2001). The high-molecular-weight (HMW) subunits of glutenin are considered to be most important for baking quality (Branlard & Dardevet, 1985; Gupta & Mac Ritchie, 1994; Kasarda, 1989) because the composition of gluten proteins is genetically determined and environmentally stable. Separation of HMW glutenin subunits is shown in Fig. 1 and Table 2.

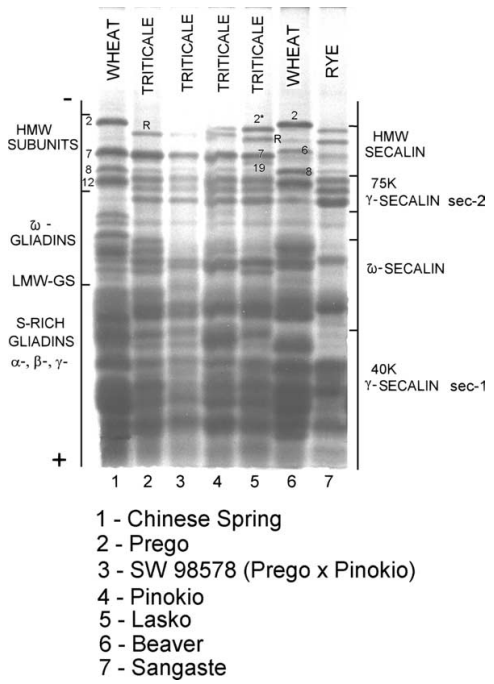

Fig. 1. SDS–PAGE of total protein fractions extracted from single seeds of wheat, rye and triticale cultivars. 1. Chinese Spring, 2. Prego, 3. SW 98578, 4. Pinokia, 5. Lasko, 6. Beaver, 7. Sangaste.

The comparison of the electrophoregram patterns of triticale cultivars with those of rye and wheat showed that most of the rye and wheat bands can be found in triticale patterns. The glutenin patterns of the cultivars Dato, Prego, and SW 98578 are more similar to the glutenin pattern of rye, whereas the glutenin pattern of Modus is closer to that of wheat.

Seven different alleles were revealed in the sample of 12 triticale cultivars: two at the *Glu-A1* locus, five at *Glu-B1*. Most frequent HMW glutenin subunits were 2* at the *Glu-A1* locus, and 7+19 at the *Glu-B1*. The genetic diversity resulting from two loci analysed enabled to distinguish 9 different patterns from 12 cultivars of triticale grown in Estonia. Most of the cultivars were uniform in their SDS electrophoretic patterns, some were heterogeneous having different alleles at the *Glu-1* locus: two variations were found for the Moreno (bands 6+8 and 7+26 at *Glu-B1*) and for Dato (bands 6+8 and 7+19 at *Glu-B1,* and N and 2* at *Glu-A1*). Polymorphism to be true for amphiploids if one of the parental species (rye) is characteristically an open-pollinating type (Virdi & Larter, 1984).

It was demonstrated that subunit 2* had a fairly good quality score, showing a slight positive correlation with the Zeleny sedimentation volume (Johansson et al., 1995). Other authors showed that subunits 1 and 2* encoded by chromosome 1A, and 6+8, 7+8 and 7+9 encoded by 1B did not correlate with strong gluten (Johansson et al., 1995; Payne et al., 1987; Uhlen, 1990). In the present investigation subunit 2* encoded by *Glu-A1,* and subunits 7+19 and 7+26 encoded by *Glu-B1* (Tables 2 and 3) were positively correlated with gluten and protein content, water absorption and bread volume. Using much more cultivars, the influence of the high-molecular-weight glutenin subunits on the technological qualities and the baking quality of triticale become clearer.

4. Conclusions

1. The baking test has shown that many cultivars of hexaploid triticale can be used in bread-making, mixing the wheat flour with up to 70% triticale amount.
2. Considering the allelic composition, the best cultivars would be Lasko, Pinokio, Prego, Dagro, Moreno, Presto, Tewo, Vision, Dato. Considering the indices characterising the bread-making properties (falling number, protein content, Zeleny number, water absorption capacity, and bread volume), Moreno, Presto, Tewo, Dato and SV 92280 were the best cultivars. This was in good accordance with the data about HMW glutenin subunit composition.
3. The baking quality of triticale cultivars was higher with HMW glutenin subunits 2* coded by the locus *Glu-A1,* and 7+26 and 7+19 coded by the locus *Glu-B1*.

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References

AACC (1983). American Association of Cereal Chemists. *Approved methods of the AACC*. Method 54-21, 10-09. St. Paul, MN: The Association.

AOAC (1984). *Association of Official Analytical Chemists* (14th ed.). Washington, DC: Official Methods of Analysis.

Branlard, G., & Dardevet, M. (1985). Diversity of grain proteins and bread wheat quality. II. Correlation between high molecular weight subunits of glutenin and flour quality characteristics. *Journal of Cereal Science, 3*, 345–354.

Branlard, G., Dardevet, M., Saccomano, R., Lagoutte, F., & Gourdon, J. (2001). Genetic diversity of wheat storage proteins and bread wheat quality. *Euphytica, 119*, 59–67.

Brzezinski, W. Lukaszewski, A.J. (1998). Allelic variation at the Glu-1, Sec-2 and Sec-3 loci in winter triticale. In *Proceedings of the fourth international triticale symposium* (Vol. 2, pp. 6–12). Canada: Red Deer Alberta.

Bushuk, W., & Larter, E. N. (1980). Triticale production, chemistry and technology. In V. Pomeranz (Ed.), *Advances in cereal science and technology* (Vol. III, pp. 115–157). St. Paul, MN: American Association Cereal Chemistry.

Cooper, K.V. (1986). Triticale food uses in Australia In *Proceedings of the first international triticale symposium of australian institute of agricultural science* (pp. 538–539).

Dervas, G., Doxastakis, G., Hadjisavva-Zinoviadi, S., & Triantafillakos, N. (1999). Lupin flour addition to wheat flour doughs and effect on rheological properties. *Food Chemistry, 66*, 67–73.

D'Ovidio, R., Lafiandra, D., & Porceddu, E. (1996). Identification and molecular characterization of a large insertion within the repetitive domain of a high molecular weight glutenin subunit gene from hexaploid wheat. *Theoretical and Applied Genetics, 93*, 1048–1054.

Doxastakis, G., Zafiriadis, J., Irakli, M., Marlani, H., & Tananaki, C. (2002). Lupin, soya and triticale addition to wheat flour doughs and their effect on rheological properties. *Food Chemistry, 77*, 219–227.

Gupta, R. B., & Ritchie, F. (1994). Mac Allelic variation at glutenin subunit and gliadinloci, Glu-1, Glu-3 and Gli-1 of common wheats II. Biochemical basis of allelic effects on dough properties. *Journal of Cereal Science, 19*, 19–29.

Hamer, R. J., Weegels, P. L., & Marseille, J. P. (1992). Prediction of the bread-making quality of wheat: The use of HMW glutenin-A subunit based quality scoring systems. *Journal of Cereal Science, 15*, 91–102.

ICC (1972, 1982). *Standard methods of the international association of cereal chemistry*. Methods No. 107, 118, 137, 167. Detmold, Germany.

Igrejas, G., Guedes-Pinto, H., Carnide, V., & Branlard, G. (1999). Seed storage protein diversity in triticale varieties commonly grown in Portugal. *Plant Breeding, 118*, 303–306.

Ingver, A., & Koppel, R. (1998). α-amylase content and its in influence on grain quality of spring wheat. In *Genetics and breeding of cereal crops* (pp. 89–96). Estonian Agricultural University.

Johansson, E. (2002). Effect of two wheat genotypes and Swedish environment on falling number, amylase activities, and protein concentration and composition. *Euphytica, 126*, 143–149.

Johansson, E., Svensson, G., & Heneen, W. K. (1995). Composition of high-molecular-weight glutenin subunits in Swedish wheats. *Acta Agriculturae Scandinavica Section B Soil and Plant Science, 45*, 112–117.

Johansson, E., & Svensson, G. (1998). Variation in bread-making quality: Effects of weather parameters on protein concentration and quality in some Swedish wheat cultivars grown during the period 1975–1996. *Journal of Science and Food Agriculture, 78*, 109–118.

Kasarda, D. D. (1989). Glutenin structure in relation to wheat quality. In V. Pomeranz (Ed.), *Wheat is unique* (pp. 277–302). St. Paul, MN: American Association Cereal Chemistry.

Kasearu, P. Laur, Ü. Vooremäe, A. Jaama, E. Kann, A. (1997). Triticale and its fields of use. In *Food and nutrition* (Vol. IV, pp. 69–79). Tallinn Technical University: Estonia.

Lafferty, J., & Lelley, T. (2001). Introduction of high molecular weight glutenin subunits 5+10 for the improvement of the bread-making quality of hexaploid triticale. *Plant Breeding, 120*, 33–37.

Laur, Ü., Jaama, E., Kasearu, P., & Vooremäe, A. (1997). Influence of sowing time on the yield and yield components of triticale. In *Collection of scientific articles. Problems of field crop husbandry and soil management in Baltic States Agronomy* (pp. 50–55). Lithuania: Kaunas.

Mac Ritchie, F., Du Cross, D. L., & Wrigley, C. W. (1990). Flour polypeptides related to wheat quality. In V. Pomeranz (Ed.), *Advances in cereal science and technology* (pp. 79–145). St. Paul, MN: American Association Cereal Chemistry.

Payne, P. I. (1987). Genetics of wheat storage proteins and the effect of allelic variation of bread-making quality. *Annual Review of Plant Physiology, 38*, 141–153.

Payne, P. I., & Lawrence, G. J. (1983). Catalogue of alleles for the complex gene loci, Glu-A1, Glu-B1, and Glu-D1 which code for high-molecular-weight subunits of glutenin in hexaploid wheat. *Cereal Research Communications, 11*, 29–35.

Payne, P. I., Nightingale, M. A., Krattinger, F., & Holt, L. M. (1987). The relationship between HMW glutenin subunit composition and breadmaking quality of British-grown wheat varieties. *Journal of Science and Food Agriculture, 40*, 51–65.

Peña, R., & Amaya, A. (1992). Milling and breadmaking properties of wheat-triticale grain blends. *Cereal Research Communications, 60*, 483–487.

Pomeranz, Y. (1985). *Functional properties of food components*. New York: Academic press, pp. 155–158.

Shewry, P. R., Popineau, Y., Lafiandra, D., & Belton, P. (2001). Wheat glutenin subunits and dough elasticity: Findings of the EUROWHEAT project. *Trends in Food Science and Technology, 11*, 433–441.

Šašek, A., Sýkorová, S., Černý, J., Hauzer, K., Kubanek, J., & Dostal, J. (1988). Electrophoretic patterns of prolamines and glutelins of some perspective cultivars of triticale. *Scientia Agriculturae Bohemoslovaca, 20*, 163–173.

Sontag-Strohm, T., & Juuti, T. (1997). Association between gliadin and glutenin subunit alleles and baking quality in spring wheat cultivars and breeding lines grown in Finland. *Acta Agriculturae Scandinavica Section B Soil and Plant Science, 47*, 106–111.

Tecator Application Note (1987). Determination of Kjeldahl Nitrogen Content with the Kjeltec Auto 1030 Analyzer. AN 30/87, 1987-09.

Tohver, M., Täht, R., Kann, A., & Rahnu, I. (2000). Investigation of seed storage protein and bread-making quality of triticale. *Acta Agronomica Hungarica, 48*(1), 41–49.

Tohver, M., Kann, A., Täht, R., & Mihhalevski, A. (2001). *The electrophoretic spectra of storage proteins and baking properties of rye, triticale and wheat. Programme and Abstracts 1st Baltic Conference on rye – in the EU-Context (Research, quality, Processing Market)*. Lithuania: Kaunas, p. 28.

Tupits, I., & Kukk, V. (1999). Results of comparison trials of triticale and winter wheat. In *From science to field and garden* (pp. 62–66). Estonian: Tartu University.

Täht, R. Kann, A. Kasearu, P. Jaama, E. Vijand, M. (1998). Baking qualities of winter triticale. In *Food and nutrition* (Vol. V, pp. 76–83). Tallinn Technical University: Estonia.

Uhlen, A. K. (1990). The composition of high molecular weight glutenin subunits in Norwegian wheats and their relation to breadmaking quality. *Norwegian Journal of Agricultural Sciences, 4*(1), 1–17.

UPOV (1994). Guidelines for the conduct of tests for distinctness, uniformity and stability. *International Union for the protection of new varieties of plants*.

Varughese, G., Pfeiffer, W., & Peña, R. J. (1996). Triticale: A successful alternative crop. *Cereal Foods World, 41*, 59.

Veskus, T. Kann, A. (1997). Falling Number as a suitability factor for determining the baking quality of rye flour. Food and nutrition (Vol. IV, pp. 95–104). Tallinn Technical University: Estonia.

Virdi, B. V., & Larter, E. N. (1984). The influence of wheat-rye species on the protein characteristics of primary triticale. *Canadian Journal Genetics and Cytology, 26*, 258–263.

Weipert, D. (1996). Mahl- und Backverhalten der Winter-Triticale-Sorten. *Pflanzenzüchtg, 34*, 49–66.

Wolski, T., (1986). Winter triticale in Polish agriculture. In *Proceedings of the 1st international triticale symposium. N.A. Darvey, compiler. Aust. Inst. Agric Sci, No. 24*.

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