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Diversity and Stability of Lactic Acid Bacteria During Rye Sourdough Propagation

ENE VIIARD

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

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Supervisor: Dr. Inga Sarand
Department of Food Processing, Tallinn University of Technology

Opponents: Dr. Åse Solvej Hansen, Associate professor
University of Copenhagen, Denmark

Dr. Fabio Minervini, Researcher
University of Bari Aldo Moro, Italy

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Declaration:

Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

Ene Viiard

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CONTENTS

INTRODUCTION

Sourdough, a mixture of flour and water that has been fermented with lactic acid bacteria (LAB) and yeast, is an intermediate product of both home baking and industrial scale bread making processes (De Vuyst and Neysens, 2005; Scheirlinck *et al*., 2008). The main purpose of fermentation is to acidify the dough which occurs as a result of the growth and metabolite production of LAB (Hansen, 2004). Leavening, however, is mainly caused by yeast via the production of carbon dioxide (Hansen, 2004).

Sourdough microorganisms are kept metabolically active by regularly adding flour and water (Hansen, 2004; De Vuyst and Neysens, 2005). In many bakeries, the sourdough is maintained for daily rye bread making by propagating it through backslopping. While the majority of the sourdough is used to make bread dough, a small portion is mixed with fresh flour and water and fermented into a new batch of sourdough. Often, daily propagation can maintain a stable microbial consortium for decades in a non-sterile bakery environment (Böcker, Vogel and Hammes, 1990; Hansen, 2004). It has been shown that so-called in-house microbiota play a key role in the stability of industrial sourdoughs (Minervini *et al*., 2012).

Studies describing various aspects of rye sourdoughs are tightly linked with the rye growing regions, which are typically in Central and Northern Europe. The microbial composition of rye sourdoughs have been characterized in Finland (Salovaara and Katunpää, 1984), Sweden (Spicher and Lonner, 1985), Denmark (Rosenquist and Hansen, 2000), Germany (Spicher and Stephan, 1993), and other neighboring regions. Until recently, very little has been known about the microbial composition of rye sourdoughs used for industrial bread making in Eastern Europe. Previous studies, many of which remain unpublished, have been performed using techniques based on phenotypic traits and biochemical characteristics. However, these approaches are often not reliable enough to identify individual LAB at species level, since different LAB species can behave in a similar manner while significant variation can occur among strains belonging to the same species (De Vuyst *et al*., 2002; Temmerman, Huys and Swings, 2004).

The aim of this work is to identify LAB in rye sourdoughs used for bread making in four Estonian bakeries that utilize different propagation parameters, with the further aim of monitoring the stability of these bacterial communities. Since rye flour is the main non-sterile component of sourdough and all four bakeries used flour from the same mill, we were able to use laboratory spontaneous sourdough fermentation to determine the availability of competent starter LAB in the commercial rye flour. This is the first study, where contemporary DNA-based methods have been used to evaluate the microbial composition of industrial rye sourdoughs produced in the Baltic countries, a region where rye bread is consumed daily and large-scale industrial rye bread production is common.

List of publications

The following publications form the basis of this dissertation and are referred to in the text by the roman numerals I through IV. They are reproduced in the appendices with permission from the publishers.

I. **Viiard, E.**, Mihhalevski, A., Rühka, T., Paalme, T. and Sarand, I. (2013) Evaluation of the microbial community in industrial rye sourdough upon continuous back–slopping propagation revealed *Lactobacillus helveticus* as the dominant species. *Journal of Applied Microbiology* 114, 404-412.

II. **Viiard, E.**, Bessmeltseva, M., Simm, J., Talve, T., Aaspõllu, A., Paalme, T. and Sarand, I. (2014) Microbial composition and stability of rye sourdoughs from four industrial bakeries. Manuscript submitted to *Journal of Applied Microbiology*.

III. Bessmeltseva, M., **Viiard, E.**, Simm, J., Paalme, T. and Sarand, I. (2014) Evolution of bacterial consortia in spontaneously started rye sourdoughs during two months of daily propagation. *PLoS ONE* 9(4): e95449. doi:10.1371/journal.pone.0095449

IV. 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.

Summary of the author's contribution

In publication I, the author carried out the analyses, interpreted the data and participated in writing the manuscript.

In publication II, the author enumerated lactic acid bacteria, extracted DNA from both isolates and sourdoughs, performed the DGGE analysis, and aided in the pyrosequencing analysis. The author also analyzed and interpreted the data and participated in writing the manuscript.

In publication III, the author participated in the experimental work and aided in pyrosequencing analysis, analyzed and interpreted the data, and participated in writing the manuscript.

In publication IV, the author isolated lactic acid bacteria used in the study, cultivated bacteria for microcalorimetry study and carried out the BioscreenC experiments.

List of conference presentations

I. FoodBalt 2010, October 2010, Tallinn, Estonia, "Population dynamics of lactic acid bacteria in industrial rye sourdough and characterization of dominant species" *(oral presentation)*

II. 4th Congress of European Microbiologists, June 2011, Geneva, Switzerland "Population composition and dynamics of lactic acid bacteria in Estonian industrial rye sourdough" *(poster presentation)*

III. 10th Symposium on Lactic Acid Bacteria, August 2011, Egmond aan Zee, The Netherlands, "Microbial composition and stability of industrial rye sourdoughs in four Estonian bakeries" *(poster presentation)*

IV. FoodMicro 2012, September 2012, Istanbul, Turkey, "Establishment of microbial consortia in semi-solid laboratory rye sourdoughs during continuous propagation at different fermentation temperatures" *(poster presentation)*

V. V Symposium on Sourdough, October 2012, Helsinki, Finland, "High-throughput sequence-based analysis of the bacterial composition of four industrial rye sourdoughs in Estonia" *(poster presentation)*

VI. 1st Congress of Baltic Microbiologists, November 2012, Riga, Latvia, "Microbial composition of rye sourdoughs determined by Rep-PCR fingerprinting, denaturing gradient gel electrophoresis (DGGE) and pyrosequencing of barcoded 16S rRNA gene amplicons" *(oral presentation)*

VII. UT and TTU graduate school „Functional materials and technologies" conference, March 2013, Tallinn, Estonia, "Analysis of microbial consortia in food– from a Petri dish to high throughput DNA sequencing" *(poster presentation)*

VIII. Terve loom ja tervislik toit 2013, March 2013, Tartu, Estonia, "Juuretise koostis ja selle mõju happesusele ja suhkrusisaldusele traditsioonilise rukkileiva tehnoloogias" *(oral presentation)*

IX. 5th Congress of European Microbiologists, July 2013, Leipzig, Germany "Metagenomic analysis gives new insight into lactic acid bacteria communities in sourdough" *(poster presentation)*

X. 11th Symposium on Lactic Acid Bacteria, August 2011, Egmond aan Zee, The Netherlands, "Development of a multi-strain rye sourdough starter" *(poster presentation)*

XI. 2nd Congress of Baltic Microbiologists, October 2014, Tartu, Estonia, "Effect of changes in propagation parameters on the microbial community of industrial rye sourdough" *(poster presentation)*

Abbreviations

CFU – colony forming unit

DGGE – denaturing gradient gel electrophoresis

DM – dry matter

DNA – deoxyribonucleic acid

DY – dough yield

FQ – fermentation quotient

HPLC – high performance liquid chromatography

HTS – high throughput sequencing

LAB – lactic acid bacteria

MRS – de Man, Rogosa and Sharpe medium

NMDS – non-metric multidimensional scaling

OD – optical density

OTU – operational taxonomic unit

PCR – polymerase chain reaction

rRNA – ribosomal ribonucleic acid

SDB – SourDough Bacteria medium

TTA – total titratable acidity

1. LITERATURE OVERVIEW

1.1 Rye bread production in Estonia

The tradition of rye bread making corresponds to the rye-growing areas in the Northern, Central, and Eastern European countries, including the Baltic States, where rye bread constitutes a considerable amount of the bread consumed (Hansen, 2004). Rye consumption in Baltic countries is traditionally very high: Estonia 19.9 kg, Latvia 14.7 kg, and Lithuania 52.3 kg per annum per capita (Valjakka, Kerojoki and Katina, 2003). Traditionally, Estonian rye bread was made from whole-grain rye flour and leavened only with sourdough without the addition of baker's yeast. According to the Estonian Association of Bakeries, during the 18th and 19th centuries home baked bread loaves weighed approximately 5–12 kg and were baked for 2–3 h. Rye bread making was laborious so up to 15 loaves of bread were prepared during one bread making session and stored for a long time. The bowls used for dough fermentation were not washed and the dried sourdough remained on the sides of the bowl and acted as a starter during the next bread making session. Since then, the size of bread loaves has gradually decreased with today's loaf weighing between 700 and 800 g. Bread is now baked more regularly and the sourdough is commonly kept active by frequent refreshments.

In today's market an increasing number of consumers insist on eating healthy traditionally fermented rye bread with an aromatic taste, good texture, and long shelf-life without the addition of food preservatives (Hansen, 2004). Organizations such as the National Institute for Health Development and bread producers from the Estonian Association of Bakeries have made an effort to educate consumers about the health benefits of whole-grain rye bread. Most of the rye bread in Estonia is still made from whole-grain rye flour, however, wheat flour is often added to improve dough handling properties, crumb structure, and taste (Valjakka, Kerojoki and Katina, 2003).

It is estimated that 150 different brands of bread are currently produced in Estonia. While rye bread loaves remain the most sold variety, rye portion breads are becoming increasingly popular. In 2012, Statistics Estonia reported the following sales of rye bread:

- 7368.4 tons of mixed rye bread (containing 50.1–89.9 % rye flour)
- 7182.3 tons of rye bread (containing over 90 % rye flour)
- 6043.1 tons fine rye breads (sweet and sour bread made with scalded flour)
- 3427.4 tons of rye bread with seeds (over 8 % of flour)
- 1913.1 tons of rye portion breads
- 667.7 tons of rye bread with different additives (fruit, nuts, meat etc.)

Generally, rye breads in the Baltic countries are characterized by a sweet and sour taste which is either obtained by adding syrup or sugar to the bread dough or results from the scalding of flour (Valjakka, Kerojoki and Katina, 2003). During scalding, the gelatinized starch hydrolyzes into dextrins and low

molecular weight sugars, which gives these breads a characteristic sweet taste. Because large quantities of sugar and high baking temperatures are used, the color of rye bread is quite dark, and sometimes almost black.

1.2 Definition of sourdough

Sourdough is a mixture of flour and water that is fermented with a microbial community dominated by lactic acid bacteria (LAB) (Hansen; 2004; De Vuyst and Neysens, 2005). The sourdough ecosystem also contains yeasts, which are primarily responsible for the leavening capacity of the sourdough (Vogel *et al*., 1996; Hansen, 2004). Sourdough propagation is typically carried out using backslopping, during which a portion of the fermented sourdough is used to inoculate a new batch of flour and water. Daily refreshment enables the microorganisms within sourdough to remain metabolically active and such frequently renewed sourdoughs can be successfully propagated for years (Hansen, 2004). However, alterations to the fermentation temperature, water/flour ratio or significant variations in the microbial composition of the flour can induce major changes in the sourdough microbiota (Valjakka, Kerojoki and Katina, 2003).

1.3 Necessity of using sourdough in rye bread production

Fermentation is an essential step in the production of traditional rye bread. The benefits of using sourdough in bread making include (Valjakka, Kerojoki and Katina, 2003; Hansen, 2004):

- leavening the dough by both gas producing LAB and yeast
- improving the rheological and gas retention properties of rye dough by inhibition of amylases and increased solubility of pentosans
- enhancing both the flavor and mouthfeel
- improving the nutritional value (higher bioavailability of minerals, lower glycemic index)
- extending the shelf-life of rye bread (about one week) by elongation of the mold-free period and anti-staling

The main purpose of sourdough fermentation in rye bread making is inhibition of enzyme activity by lowering the pH. The activity of α-amylase, a starch degrading enzyme, is higher in rye compared to other cereals (Seibel and Weipert, 2001) and has optimum activity at pH 5.5. However, the enzyme is completely inactive at pH values below 4.0 (Seibel and Weipert, 2001). This prevents an excessive release of maltodextrin from the rye starch during bread making. Dextrin and low molecular weight sugars can give the bread crumb an undesirable sticky texture that drastically affects the quality of rye bread.

Lowering the pH of rye dough is required to obtain a final product with the desired porosity and volume. Rye proteins differ from wheat proteins as they do not form a gluten matrix that gives texture and volume to wheat bread (Kratochvil and Holas, 1984). Thus, proteins have a minor role in rye dough

rheology and bread texture. Pentosans, 85–90 % of which are arabinoxylans, are responsible for the viscosity of rye dough due to their high water-binding capacity (Valjakka, Kerojoki and Katina, 2003). The content of arabinoxylans is higher in rye (6–8 %) compared to wheat which contains only up to 2 %. The solubility, water-binding and swelling capability of pentosans is higher in acidic environments (Hammes and Gänzle, 1998; Martines-Anaya and Devesa, 2000). Thus, in addition to giving rye bread good porosity and crumb structure, the production of lactic acid by sourdough LAB provides dough with better rheological properties and gas retention capacity.

1.4 Chemical characteristics of sourdough

The quality of sourdough bread depends to a great extent on the stability of chemical attributes of the sourdough. In order to obtain sourdough with invariable quality from each fermentation cycle, parameters such as fermentation temperature and time, initial pH, inoculum size, and type of fermentation system (batch or continuous) must be meticulously controlled (Hansen, 2004). Sourdoughs are characterized by their main chemical properties– pH, total titratable acidity (TTA), and the concentration of both lactic and acetic acid (Hansen, 2004).

1.4.1 pH and total titratable acidity

The final pH of a mature fermented rye sourdough is typically between 3.5 and 4.3, depending in its further application in bread making (Lund, Hansen and Lewis, 1989; Bruemmer and Lorenz, 1991; Hansen and Hansen, 1994; De Vuyst and Neysens, 2005). If the ratio of sourdough used in the bread dough is low, a lower pH value is required, with the converse also being true. In contrast with pH, the range of typical TTA values is much wider (Hansen, 2004). The TTA of sourdough characterizes the concentration of weak organic acids (such as lactic and acetic acid). This value depends on the fermentation temperature, extraction rate of the flour and dough moisture content. The higher the extraction rate, the more of the bran, germ and outer layers of endosperm the flour contains. In wheat sourdoughs, TTA values typically range between 8–11, if low extraction rate flour is used, and 16–22 in whole-grain sourdoughs (Bruemmer and Lorenz, 1991; Hansen and Hansen, 1994). Rye sourdoughs are commonly made from flours with a high extraction rate and thus TTA values typically fall within the range of 15–26 (Lund, Hansen and Lewis, 1989; Spicher and Stephan, 1993; Hansen, 2004).

1.4.2 Lactic and acetic acid ratio

The concentration of lactic and acetic acid in bread dough is very important for the taste and aroma of the final product (Hansen, Lund and Lewis, 1989; Hansen and Hansen, 1996; Valjakka, Kerojoki and Katina, 2003). The

fermentation quotient (FQ), measured as the molar ratio between lactic and acetic acid, is used to describe the balance between these two acids (Spicher and Stephan, 1993). In order to give bread a balanced taste and aroma profile, the ratio of lactic acid to acetic acid should be about 4:1. If the concentration of acetic acid is low, the bread has too little flavor and lacks characteristic aromas, if the relative amount of acetic acid is too high, the taste and aroma can be sharp and pungent (Spicher and Stephan, 1993). Considering these aspects, the concentration of acetic acid should be kept quite low, however, it has also been shown that acetic acid is more efficient in preventing the growth of mold (Rosenquist and Hansen, 1998). Thus, it is important to maintain the ratio between these two organic acids. The production of lactic and acetic acid is greatly influenced by both the composition of the LAB community and sourdough fermentation parameters.

1.5 Approaches for obtaining rye sourdough

Sourdoughs can be started by:

- Spontaneous fermentation of rye flour
- Inoculation with mature sourdough from previous fermentation
- Adding defined starter cultures to initiate fermentation

1.5.1 Spontaneous flour fermentation

Spontaneous sourdough fermentation is initiated by mixing flour with water and allowing the flour microbiota to ferment the dough for up to two days. However, spontaneous sourdough fermentations do not always succeed and may not result in a final product with desirable taste, aroma, and texture. Therefore, this is neither considered a reliable nor practical approach to obtain sourdough within the baking industry. Spontaneous fermentation is typically only carried out to initiate the first step of a new sourdough cycle. Repeated re-inoculation of a fresh flour and water mixture from a previously fermented sourdough is more commonly carried out on a daily basis to obtain a mature sourdough (Hansen, 2004; De Vuyst and Neysens, 2005; De Vuyst *et al*., 2014).

Flour is abundant in carbohydrates and the sourdough has an initial pH of 5.0–6.2, which allows the growth of microorganisms originating from flour (De Vuyst and Neysens, 2005). Spontaneously started and repeatedly backslopped sourdough fermentations are characterized by a three-phase successive evolution of the microbial communities and usually result in a mature sourdough within a week (Van der Meulen *et al*., 2007; Weckx *et al*., 2010a; Weckx *et al*., 2010b; Weckx *et al*., 2011). While flour is dominated by Gram-negative bacteria, Gram-positive LAB start to prevail when the pH of the sourdough drops from its initial level during the first renewal cycles (Lönner and Preve, 1988; Spicher and Stephan, 1993; Hansen, 2004; Ercolini *et al*., 2013). Sourdough non-specific LAB initially dominate the microbial community (e.g. lactococci and enterococci), however, the increase in acidity causes them to be gradually

replaced by sourdough characteristic LAB species (e.g. species of the genera *Weissella, Lactobacillus,* and *Leuconostoc*). During the final stage of sourdough maturation, highly adapted sourdough specific LAB, mainly belonging to the genus *Lactobacillus*, become prevalent (Van der Meulen *et al*., 2007; Weckx *et al*., 2010b; Minervini *et al*., 2014). These acid-tolerant species are usually able to metabolize a wide range of cereal carbohydrates and thereby have an advantage in the sourdough environment. It has been shown that in rye sourdough this three-stage evolution occurs more rapidly compared with wheat and spelt sourdoughs, which may be due to the high enzymatic activity in rye flour that provides substrates for the LAB (Van der Meulen *et al*., 2007; Weckx *et al*., 2010b).

1.5.2 Inoculation with mature sourdough

Most sourdoughs used by artisan bakers and small scale bakeries have been initiated by spontaneous fermentation after which the sourdough has been kept metabolically active and stable for years by daily refreshment (Figure 1).

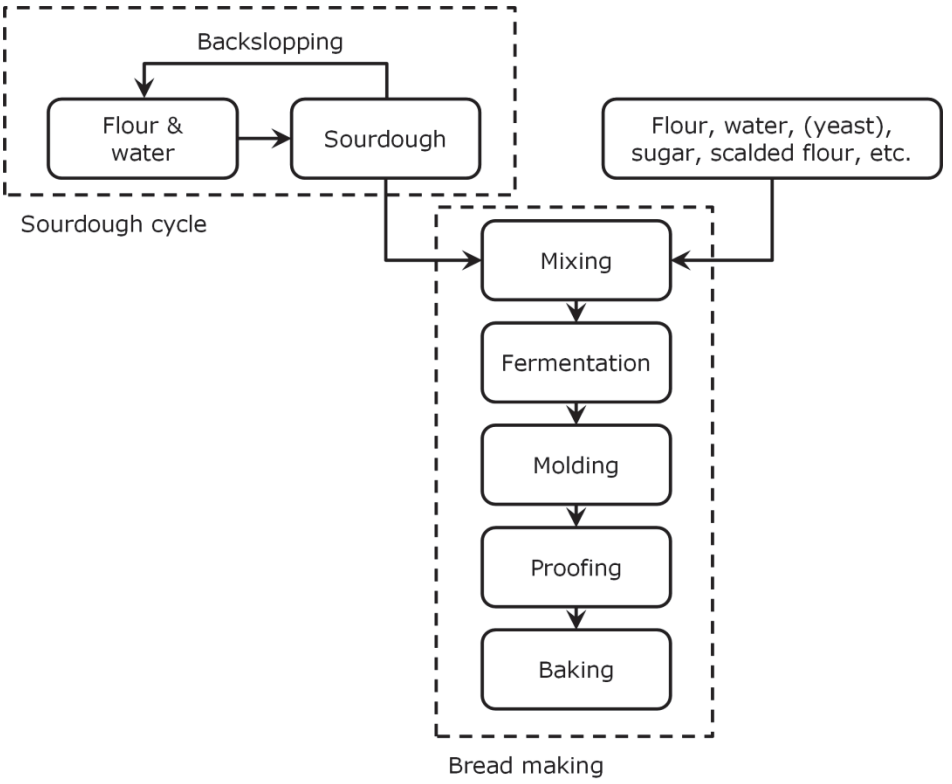

Figure 1. Sourdough propagation cycle using mature sourdough to inoculate a new batch of sourdough and simplified rye bread making process common in Estonian bakeries.

The majority of the fermented sourdough is utilized during bread making and a small portion of the mature sourdough is used as a starter by mixing it with flour and water to initiate fermentation of a new batch of sourdough (Hansen, 2004). Although the bakery environment and equipment are far from sterile, the microbial composition of these sourdoughs can remain remarkably stable for years. It has been shown that the in-house microbiota of the bakery play a key role in the stability of sourdoughs (Minervini *et al*., 2012).

1.5.3 Inoculation with defined starter cultures

Freeze-dried cultures of single strains or combinations of lactobacilli and yeasts are commercially available (Hammes, Stolz and Gänzle, 1996; Hansen, 2004). The freeze-dried powders are mixed with flour and water, and fermented for several hours to allow the starter bacteria to grow, multiply and produce acid. The microorganisms used as starters have been selected due to their ability to acidify the dough in a short period of time and result in bread with acceptable taste and aroma. However, these strains may not be adapted to the selected sourdough fermentation parameters and are therefore unable to compete with the spontaneously growing microflora entering the sourdough with raw materials or from the bakery environment (Hammes and Gänzle, 1998; De Vuyst and Neysens, 2005; Minervini *et al*., 2010; Moroni *et al*., 2010). Therefore, further repeated backslopping propagation of sourdough made with commercial starter cultures is generally not possible.

1.6 Types of sourdoughs

Sourdoughs have been grouped into three types, based on the production technology (Böcker, Stolz and Hammes, 1995):

- type I sourdoughs or traditional sourdoughs
- type II sourdoughs or accelerated sourdoughs
- type III sourdoughs or dried sourdoughs

1.6.1 Type I sourdoughs

Type I sourdoughs are fermented at room temperature and renewed regularly to keep the microorganisms metabolically active. They are characterized by low moisture content and therefore have a firm structure (De Vuyst and Neysens, 2005; De Vuyst *et al*., 2014). Fermentation is carried out at ambient temperature (20–30°C) and the pH of mature sourdough is approximately 4.0 (Stolz, 1999; De Vuyst and Neysens, 2005). The fermentation time can vary drastically taking between 3–48 h. Type I sourdoughs are widely used by artisan bakers and small-scale bakeries.

1.6.2 Type II sourdoughs

Type II sourdoughs are propagated at temperatures above 30°C using industrial scale temperature-controlled containers (De Vuyst and Neysens, 2005; De Vuyst *et al*., 2014). Type II sourdough is commonly semi-liquid to suit the material handling processes applied in large scale production facilities to ferment sourdough faster using more controllable methods. Handling large portions of semi-liquid sourdough during bread making and renewal is easier because it allows one to stir and pump the sourdough. This type of sourdoughs can be continuously propagated and also long-term one-step fermentations are common; this increases both the reliability and flexibility of production (De Vuyst and Neysens, 2005). Type II sourdoughs can be fermented for several days (Böcker, Stolz and Hammes, 1995; Hammes and Gänzle, 1998) during which the pH drops below 3.5 within the first 12–24 h of fermentation. At this point the microorganisms are in the late stationary phase and their metabolic activity is restricted.

1.6.3 Type III sourdoughs

Type III sourdoughs are dried dough acidifiers and flavor enhancers and contain LAB species that are resistant to drying (Stolz and Böcker, 1996; De Vuyst and Neysens, 2005; De Vuyst *et al*., 2014). They can be easily used to achieve standardized final products. While type I sourdoughs can be used without baker's yeast because they contain significant numbers of yeasts, type II and III sourdoughs require additional gas production by yeast added to the sourdough to leaven the bread dough prior to baking (De Vuyst and Neysens, 2005; De Vuyst *et al*., 2014).

1.7 Parameters affecting the properties of sourdough

The outcome of sourdough fermentation can be adjusted by altering propagation parameters: flour type, dough yield (DY), fermentation temperature and time, and the amount of starter used for inoculation (Spicher and Stephan, 1993; Hansen, 2004). The combined effect of the selected propagation parameters qualitatively and quantitatively influences the dominant sourdough microbiota, which in turn defines the outcome of the fermentation process (Minervini *et al*., 2014).

Traditional rye sourdough used in both home baking and small bakeries has a firm structure, however in automated large-scale bakeries dough with thick consistency is difficult to handle. To counter this, semi-fluid or fluid sourdoughs have been developed for use in automated fermentation systems. The water content determines the firmness of the sourdough and is commonly expressed as DY, which is the amount of sourdough in kg obtained per 100 kg of flour. DY can vary from 150 in firm sourdoughs used in artisan bakeries to 400 in fluid sourdoughs characteristic for large-scale production (Hansen, 2004).

It has been shown that firm sourdoughs are prone to fluctuations in both microbial and chemical characteristics (Di Cagno *et al*., 2014). More rapid acidification in high DY sourdoughs allows acid tolerant LAB to dominate (Decock and Cappelle, 2005). The LAB counts are more stable in constantly stirred sourdoughs due to better availability of carbohydrates and other nutrients (Di Cagno *et al*., 2014). Although sourdoughs with low DY have a higher concentration of available substrates, nutrients are better utilized by LAB in liquid sourdoughs which are usually subjected to constant stirring assuring an even distribution of substrates (Vogelmann and Hertel, 2011; De Vuyst *et al*., 2014). The production of acetic acid is generally lower in liquid sourdoughs (Lund, Hansen and Lewis, 1989; Spicher and Stephan, 1993).

The redox potential is mainly determined by the amount of oxygen incorporated in the sourdough during mixing. The availability of oxygen can provide an advantage to those microorganisms that are able to use it as an electron acceptor (De Vuyst and Neysens, 2005; Minervini *et al*., 2014). However, it has been shown that many LAB are aero-tolerant or microaerophilic and do not require the presence of oxygen (Hammes and Vogel, 1995).

Fermentation temperature is the key factor that influences the composition of microbial communities in sourdough and also determines metabolite production (De Vuyst *et al*., 2014). The ratio of lactic acid and acetic acid is shifted toward the production of lactic acid at higher fermentation temperatures. Low fermentation temperatures support the growth of yeast and thereby induce formation of flavor compounds, ethanol and carbon dioxide. If acetic acid and yeast growth is desired, fermentation temperature should be kept between 20–25°C, whereas to encourage lactic acid production, fermentation temperature in the range 32–38°C should be applied (Valjakka, Kerojoki and Katina, 2003; Minervini *et al*., 2014), since 30–35°C is the optimal growth temperature for most LAB species (Spicher and Stephan, 1993). Usually the growth optimum of LAB is also the temperature at which most lactic acid is produced. Generally the final pH is achieved more quickly at higher fermentation temperatures compared to lower temperatures (Hansen, Lund and Lewis, 1989; Brummer and Lorenz, 1991; Spicher and Stephan, 1993). Taking all this into account, the total amount of acid produced in sourdough increases with temperature, although most of this is lactic acid because the production of acetic acid is only marginally affected by fermentation temperature (Hansen, Lund and Lewis, 1989; Spicher and Stephan, 1993). The relative content of acetic acid is therefore higher in sourdoughs fermented in colder conditions. This can significantly influence the flavor profile of the final product.

In small bakeries it is common that the sourdough is kept refrigerated between bread makings. At lower temperature the fermentation slows down, but the microorganisms remain viable (Valjakka, Kerojoki and Katina, 2003). This procedure can shift the microbial community towards favoring cold-tolerant species that can manage the stress caused by low temperatures and starvation (Mantynen *et al*., 1999; Siragusa *et al*., 2009). Formerly it was common that the bakers and artisanal bakeries did not wash the bowls used for sourdough

fermentation. The remains of sourdough dried in the bowl and initiated the next fermentation (Valjakka, Kerojoki and Katina, 2003). Following this principle it is still common in small-scale bakeries to mix sourdough with flour to lower the water activity during storage at room temperature, which should prevent the growth of microorganisms until the beginning of a new fermentation cycle. However, it is not completely clear how this procedure affects the microbial community of the sourdough.

The ratio of inoculum to flour and water mixture determines the initial pH of the newly mixed sourdough and thereby directly influences the growth of sourdough LAB and yeasts (Van der Meulen *et al*., 2007). The suggested amount of mature sourdough used for inoculation of flour and water commonly varies between 10–20 % (Brummer and Lorenz, 1991; Spicher and Stephan, 1993). However, the inoculation rate can also be remarkably higher– San Francisco sourdough is refreshed at least two to three times a day and inoculum is 25–40 % of the sourdough (Kline, Sugihara and McCready, 1970). The use of high percentages of inoculum inhibits the growth of lactobacilli, thereby providing an advantage to yeasts (Brandt, Hammes and Gänzle, 2004). In common practice the longer the fermentation time, the smaller the inoculum. Short cycles favor fast growing and acid producing LAB species and yeast (Vogelmann and Hertel, 2011) while longer fermentations without renewal are suitable for acid-tolerant LAB that are accustomed to nutrient depletion (Van der Meulen *et al*., 2007).

1.8 Microbial communities in sourdough

Sourdoughs are a vast source of LAB and yeast species due to their artisan handling (De Vuyst and Neysens, 2005; De Vuyst *et al*., 2014). More than 50 species of LAB and more than 20 species of yeasts have been identified in sourdough (De Vuyst and Neysens, 2005). Main genera of LAB identified from sourdoughs are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Weissella*. The majority of the sourdough LAB belong to the genus *Lactobacillus* (Hansen, 2004). Most commonly found yeast are species of *Saccharomyces* and *Candida*. The number of LAB and yeast in sourdoughs is 10^8–10^9 CFU g^{-1} and 10^6–10^7 CFU g^{-1}, respectively (Spicher and Stephan, 1993; Vogel *et al*., 1996).

1.8.1 Metabolism of sourdough lactic acid bacteria

Sourdoughs are dominated by LAB– a group of Gram-positive bacteria that are catalase-negative, non-motile, non-sporeforming rods or cocci that produce mainly lactic acid during the fermentation of carbohydrates. They are strictly fermentative, typically aerotolerant or micro-aerophilic, acidophilic, salt-tolerant, and have complex nutritional requirements for carbohydrates, amino acids, peptides, fatty acids, salts, nucleic acids derivatives, and vitamins (Hammes and Vogel, 1995; Gänzle, Ehmann and Hammes, 1998).

From the baker's point of view, the main task of sourdough lactobacilli is to produce lactic acid from cereal carbohydrates. The most prevalent genus, *Lactobacillus,* has been divided into three groups according to their carbohydrate fermentation patterns (Kandler and Weiss, 1986):

1) Obligately homofermentative LAB

Hexoses are almost completely fermented to lactic acid (>85 %) by the Embden-Meyerhof-Parnas (EMP) pathway. Fructose is also fermented, but pentoses are not utilized.

2) Facultatively heterofermentative LAB

Hexoses are almost completely fermented to lactic acid by the EMP pathway. Pentoses are fermented to lactic acid and acetic acid by phosphoketolase pathway.

3) Obligately heterofermentative LAB

Hexoses are fermented to lactic acid, acetic acid (ethanol) and carbon dioxide. Pentoses are fermented to lactic and acetic acid. Both pathways involve phosphoketolase.

Generally homofermentative LAB rapidly produce high concentrations of lactic acid at higher fermentation temperatures. Heterofermentative LAB species dominate at lower fermentation temperatures with longer fermentation times and, aside from lactic acid, they produce large amounts of acetic acid, and/or ethanol. Some species are capable of producing high volumes of carbon dioxide and thereby also leaven the dough (Hansen, 2004; De Vuyst *et al*., 2014). The latter feature is especially important in the manufacture of traditional rye bread, to which baker's yeast is often not added due to consumer demand.

Starch, the main polysaccharide in flour, is generally not degraded by LAB. The content of fermentable mono- and disaccharides in rye flour can reach levels of 5 %, with maltose (3 %) as the main sugar (Savola, Salovaara and Enqvist, 1983). However, during enzymatic degradation of starch, maltose is continuously released and thereby available for the maltose-positive LAB and yeasts to utilize. In most fermented foods homofermentative LAB play a key role, while heterofermentative LAB dominate in the microbial communities of sourdough (De Vuyst *et al*., 2002; Corsetti *et al*., 2003). Obligately heterofermentative lactobacilli are quite capable of fermenting the main cereal carbohydrates (e.g. the fermentation of maltose by *Lactobacillus fermentum, Lactobacillus reuteri,* and *Lactobacillus sanfranciscensis*) (Gobbetti *et al*., 2005; Gänzle, Vermeulen and Vogel, 2007; De Vuyst *et al*., 2014).

1.8.2 Lactic acid bacteria species characteristic to sourdough

Sourdough microflora include well-adapted LAB that have optimal growth and fermentation conditions within sourdough, and are likely to produce antimicrobial compounds that suppress the growth of competing organisms (Messens and De Vuyst, 2002; Hansen, 2004). According to Weckx *et al*. (2010b), differences in the chemical composition of the raw materials, rye and

wheat sourdough fermentations are similar and do not significantly differ in their microbial composition. Species *Lactobacillus plantarum*, *Lactobacillus brevis*, *L. sanfranciscensis*, *L. fermentum*, and *Leuconostoc mesenteroides* are frequently found in wheat sourdoughs and *L. plantarum*, *L. brevis*, *L. fermentum*, and *L. sanfranciscensis* prevail in rye sourdoughs, thus confirming the similarity of sourdoughs made from these two cereals (De Vuyst *et al*., 2014).

Rather than flour origin (wheat or rye), the composition of LAB depends on the type of sourdough. The microbial community of type I sourdoughs frequently includes species such as obligate heterofermentative strains of *L. sanfranciscensis*, *L. brevis* and the related *Lactobacillus buchneri*, *L. fermentum*, *Lactobacillus fructivorans*, *Lactobacillus pontis*, *L. reuteri*, and *Weissella cibaria*, facultative heterofermentative *Lactobacillus alimentarius*, *Lactobacillus casei*, *Lactobacillus paralimentarius*, and *L. plantarum*, and obligate homofermentative *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus farciminis*, and *Lactobacillus mindensis* (Hammes and Gänzle, 1998; Vogel *et al*., 1999; Minervini *et al*., 2014). The main task of these bacteria in sourdough is the production of acids and carbon dioxide. Unlike type I sourdoughs, *L. sanfranciscensis* is not able dominate under type II fermentation conditions, where one tends to find obligate homofermentative and acid tolerant *L. acidophilus*, *L. delbrueckii*, *Lactobacillus amylovorus*, *L. farciminis*, and *Lactobacillus johnsonii*, and obligate heterofermentative *L. brevis*, *L. fermentum*, *Lactobacillus frumenti*, *L. pontis*, *Lactobacillus panis*, *L. reuteri*, as well as *Weissella* species (Vogel *et al*., 1999; Müller *et al*., 2001). A selection favoring heterofermentative LAB usually occurs during sourdough renewal and is determined by backslopping parameters (inoculum size, renewal frequency and number of refreshments) (Minervini *et al*., 2012; De Vuyst *et al*., 2014).

1.8.3 Microbial stability of rye sourdoughs

The influence of sourdough propagation parameters during repeated backslopping both triggers the selection of characteristic LAB and yeast species and prevents the growth of other microorganisms that originate from raw materials (De Vuyst and Neysens, 2005). Despite changes in the chemical and microbial composition of raw materials, the microbial communities of sourdough can be remarkably stable. Monitoring of the microbial community of two industrial Danish sourdoughs over seven months revealed only minor changes (Rosenquist and Hansen, 2000). LAB species that are well-adapted to sourdough fermentation conditions can remain dominant for a long time during continuous propagation of type II sourdoughs and are enriched in the sourdough environment (Hammes and Gänzle, 1998; Meroth *et al*., 2003). However, the microbial community can change significantly when and if the sourdough propagation conditions are drastically altered. In bakery environment the in-house microbiota play a key role in stabilizing the microbial communities in sourdough by acting as an inoculum during each refreshment step. If this

environment is significantly changed, remarkable shifts can occur in the community (Minervini *et al*., 2012).

1.9 Enumeration and identification of lactic acid bacteria

Initially it was presumed that a link exists between the geographical region of the sourdough and its microbial community, however, this apparent specificity is now attributed to differences in the methods used for isolation, characterization and identification. Therefore, to ensure reproducibility, both the sampling procedure and identification steps are of critical importance (De Vuyst and Neysens, 2005; De Vuyst and Vancanneyt, 2007; Huys, Daniel and De Vuyst, 2013; De Vuyst *et al*., 2014).

It has been found that many sourdough LAB are difficult to cultivate using common laboratory media. The reason for this may be that the bacteria have been selected during repeated sourdough propagation which results in a community with specific nutrient and growth requirements (Kline and Sugihara, 1971; Salovaara and Katunpää, 1984; Böcker, Vogel and Hammes, 1990; Rosenquist and Hansen, 2000). Previously, identification of LAB species was mainly performed using methods based on colony morphology and biochemical traits. Modern identification of isolated LAB combines phenotypic and genotypic methods, with the latter often utilizing polymerase chain reaction (PCR) and 16S rRNA gene sequencing (Figure 2) (De Vuyst and Vancanneyt, 2007).

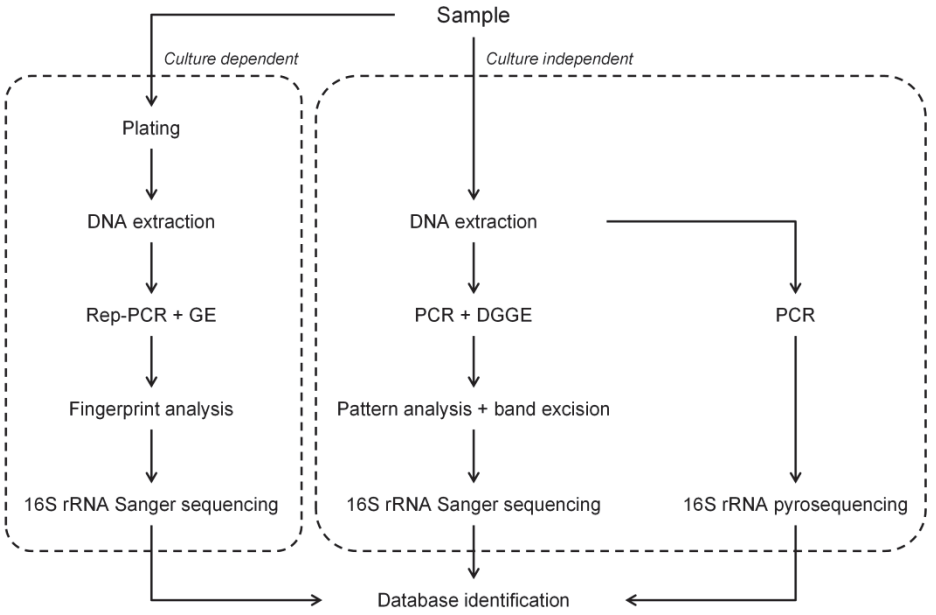

Figure 2. Culture-dependent and culture-independent methods commonly applied to study microbial communities in sourdoughs.

Culture-dependent approach (selective plating and incubation followed by identification with molecular techniques) is performed in parallel with culture-independent analyses (denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene amplicons or metagenomic sequencing of barcoded 16S rRNA gene amplicons of whole DNA isolated from the sample) in order to determine composition of microbial communities (Figure 2) (Van der Meulen *et al*., 2007; Weckx *et al*., 2010b; Ercolini, 2013; De Vuyst *et al*., 2014). Although suitable for community fingerprinting, culture-independent methods do not always provide accurate quantitative information (De Vuyst and Vancanneyt, 2007). It should also be noted that there is no universal method that can be used to identify all LAB species.

1.9.1 Culture-dependent methods

Some organisms grow poorly on laboratory media, and therefore may escape isolation and cannot be identified using standard procedures (Hansen, 2004). It has further been shown that there is no universal medium suitable for a comprehensive study of sourdough species (Vera, Rigobello and Demarigny, 2009). Isolation of LAB from sourdough depends on both the composition of the medium and incubation conditions (De Vuyst and Vancanneyt, 2007). The choice of media is critical for it affects both the quantitative and qualitative nature of the results. MRS (de Man, Rogosa and Sharpe), maltose MRS, SDB (SourDough Bacteria), SFM (Sanfrancisco medium), MRS "Vogel", MRS5 and Rogosa media have all been used to cultivate sourdough lactobacilli and a combination of these media is required to estimate the overall microbial diversity (Vera, Rigobello and Demarigny, 2009). Despite this knowledge, most studies published to date have utilized only one or two selected media.

The isolation of microorganisms from sourdough using culture-dependent techniques implicitly assumes that the dominant organisms are all culturable. Hence, one requires prior knowledge about the possible composition of the sourdough community to obtain reasonable results. This understanding is a prerequisite for the selection of appropriate media and cultivation conditions (including nutrients in the media, pH, temperature and atmosphere) (De Vuyst and Vancanneyt, 2007; Huys, Daniel and De Vuyst, 2013; De Vuyst *et al*., 2014). Colony isolation is typically followed by fingerprint typing using specific primers to amplify repetitive elements (Rep-PCR) of the bacterial DNA (Temmerman, Huys and Swings, 2004). Such primers include REP, ERIC, BOX and $(GTG)_5$. It has been shown that $(GTG)_5$ can differentiate between LAB species possibly even at the strain level (Gevers, Huys and Swings, 2001; Temmerman, Huys and Swings, 2004). Fingerprinting is commonly followed by sequencing of the 16S rRNA gene amplicons to identify the isolates. To obtain a more complete overview of the diversity of microbial communities and their community dynamics within food matrices, it is recommended to use agar media in combination with culture-independent methods (Vera, Rigobello and Demarigny, 2009).

1.9.2 Denaturing Gradient Gel Electrophoresis of rRNA gene amplicons

Culture-independent methods are based on the analysis of metagenomic DNA isolated from the food sample. Methods such as DGGE of PCR-amplified partial 16S rRNA gene fragments provide a better understanding of the dynamics of microbial communities in sourdoughs. DGGE analysis is one of the most convenient and widely used approaches used to study bacterial communities from various environments (Muyzer, 1999; Meroth *et al*., 2003; Temmerman, Huys and Swings, 2004). This method allows one separate a mixture of DNA fragments with identical length but different sequence obtained by PCR amplification using universal primers (Temmerman, Huys and Swings, 2004). In an acrylamide gel containing a gradient of denaturing components, amplicons with different sequences unwind depending on their melting domains (Figure 3).

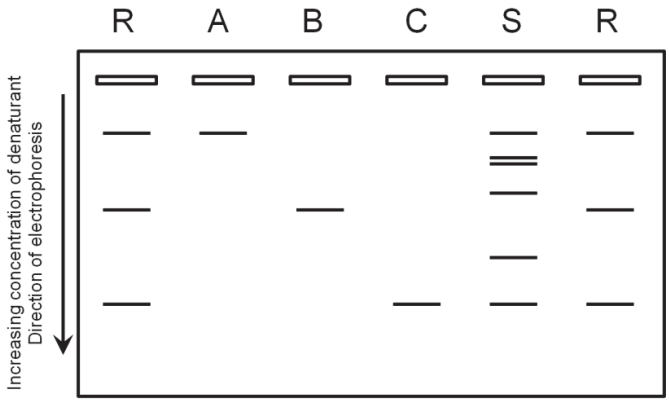

Figure 3. Denaturing gradient gel electrophoresis (DGGE). R – reference sample; A – organism 1; B – organism 2; C – organism 3; S – unknown sample. Adapted from Temmerman, Huys and Swings (2004).

A GC-clamp is attached to the 5'-end of at least one of the primers to prevent the amplicons from completely denaturing. 16S rRNA gene amplicons with different melting domains, as determined by their GC content, stop at different positions in the gel. Bands of interest observed on DGGE can be excised and sequenced to identify the members of the community.

PCR-DGGE allows one to monitor the dynamics of microbial populations and further enables one to reveal non-cultivable organisms that remain undetected using plating techniques (Meroth *et al*., 2003; De Vuyst and Vancanneyt, 2007). The primers used for PCR amplification prior to DGGE are of critical importance. The use of universal primers that target the V3 or V6-V8 region of the 16S rRNA gene enables amplification of most species within bacterial communities (Temmerman, Huys and Swings, 2004). However, some species may not be detected by PCR-DGGE, although they are found using

cultivation based methods. This can largely be explained by differences in DNA extraction efficiency from various species and/or the fact that some DNA templates can be better amplified (Meroth *et al*., 2003). Also, bacteria that are present at low numbers and form small subpopulations are usually not revealed by DGGE. This method does, however, allow one to discriminate between the more abundant species in the microbial community. To detect less abundant groups in the sample, group-specific primers should be used (Temmerman, Huys and Swings, 2004). Other weaknesses of this approach are the occurrence of multiple 16S rRNA operons that may provide multiple bands for a single species, strain-specific detection limit, and inability to quantify the results because band intensity does not correspond with the total count of the species in the food sample (De Vuyst and Vancanneyt, 2007). DGGE also may not be able to discriminate between closely related microorganisms, due to short amplicon length. For these reasons DGGE should be combined with culture-dependent methods to obtain total microbial counts together with the diversity of culturable bacteria and thereby gain an overview of the microbial community composition (De Vuyst and Vancanneyt, 2007).

1.9.3 High-throughput sequencing of metagenomic DNA

Culture-independent evaluation of microbial diversity in food can be performed by using high-throughput sequencing (HTS) approaches after extracting DNA directly from the samples (Ercolini, 2013; De Vuyst *et al*., 2014). Other culture-independent methods (such as DGGE) are low throughput compared to HTS and do not reveal species belonging to subpopulations (Illeghems *et al*., 2012; Ercolini *et al*., 2013; De Vuyst *et al*., 2014). The proportion of organisms among the entire food microbiome is usually determined by sequencing the rRNA gene amplicons. The sequences obtained using this method can be grouped into operational taxonomic units (OTUs) based on their similarity and then compared to reference databases in order to identify the taxa (Ercolini, 2013). The relative abundance of each OTU in the sample is determined by the number of sequences that belong to the same OTU. Although sequencing the rRNA gene amplicons does provide an overview of which organisms are present, shotgun metagenomics allows one to observe the possible metabolic activities of those organisms by sequencing microbial genes (Jung *et al*., 2011; Illeghems *et al*., 2012; Ercolini, 2013; De Vuyst *et al*., 2014). This is especially crucial when studying food systems, where the metabolic activity of organisms can vary during different stages of fermentation, such as cheese ripening. However, within sourdoughs the majority of microorganisms are metabolically active as a result of repeated refreshment.

Depending on the sequencing technology utilized, the results obtained using HTS can vary in their sample coverage (determined by number of sequences) and taxonomic resolution (determined by sequence length) (Ercolini, 2013). In raw materials such as rye flour, the microbiota can be quite complex due to contamination from soil, mills, insects, and other contaminants. In contrast, the

microbial diversity within sourdough is quite narrow and the fermentation process is dominated by a very small number of well adapted species. In order to obtain relevant information about the microbial composition of sourdoughs, identification at species level is needed. Short sequences only provide discrimination at the family or genus level, however, it is common knowledge that the majority of bacteria in sourdough communities belong to the genus *Lactobacillus*. Longer sequences that include data from more 16S rRNA variable regions make it possible to discriminate between closely related species. However, even in this case the 16S rRNA is not always heterogeneous enough to distinguish between different species (Ercolini, 2013).

Metagenomic HTS is considered a quantitative approach as there is a relationship between the number of organisms in the sample and the quantity of DNA, number of amplicons, and number of sequences belonging to that organism (Ercolini, 2013). However, DNA extraction must be optimized to prevent bias during the extraction process so that one may obtain comparable results from the majority of microbes in the sample.

2. AIMS OF THIS THESIS

The aims of this thesis are to:

I. Assess the possibility of using a freeze-dried mature sourdough as a starter in a new industrial sourdough fermentation and evaluate the stability of the microbial community during continuous propagation (Publication I)

II. Identify prevalent lactic acid bacteria communities of rye sourdoughs in four Estonian bakeries with different propagation parameters and monitor the microbial stability of the sourdoughs (Publications I and II)

III. Determine the availability of competent starter LAB in commercial rye flour used for bread making in most Estonian bakeries (Publication III)

IV. Characterize the carbohydrate fermentation profile and evaluate the effect of oxygen on the growth of cereal-adapted *Lactobacillus helveticus* – a LAB species not common in rye sourdough (Publications I and IV)

3. MATERIALS AND METHODS

Detailed descriptions of the materials and methods used are available in Publications I to IV.

3.1 Collection of sourdough samples

3.1.1 Industrial sourdough samples

Sourdoughs from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) were studied. Two of the sourdoughs can be roughly classified as type II (A_{bakery} and B_{bakery}) and two are type I (C_{bakery} and D_{bakery}). All bakeries used flour from the same origin (Tartu Mill AS, Estonia) and applied the sourdough propagation parameters presented in Table 1.

In A_{bakery} the sourdough process is initiated with a freeze-dried sourdough (A0) previously prepared from a sourdough used for bread making in the same bakery. The renewal of this industrial type II sourdough was initiated by mixing the freeze-dried sourdough starter with rye flour and tap water. Sourdough is routinely backslopped and fermented at 32°C for 10 h (Table 1). The fermentation process is strictly controlled and renewals are carried out at regular time intervals. Publication I presents a more detailed description of the full set of samples collected from A_{bakery} for the purpose of studying the possibility of using a freeze-dried mature sourdough to initiate a new industrial sourdough fermentation.

The sourdough in B_{bakery} is also a type II liquid sourdough used in large scale rye bread production. The sourdough in use (Bs) had been successfully propagated for three years and was then renewed from fresh active sourdough (B0) obtained from another bakery. The sourdough is fermented at 42–44°C for 16 h (Table 1) in a strictly monitored process.

The sourdough in C_{bakery} was a type I sourdough used in small scale production. The fermentation of this sourdough was originally initiated with a commercial starter (C0) one year before the first sample was collected. The sourdough was propagated at ambient temperature, and refreshed every 4 h (Table 1). Unfortunately C_{bakery} stopped producing rye bread after the third sample (C3) was collected and thus data obtained for C_{bakery} sourdough is limited.

D_{bakery} sourdough also uses a type I sourdough for small scale production. Their sourdough dates back to the 1980's and has been successfully propagated for over 30 years. A commercial starter (probably the same starter used in C_{bakery}) was originally used to initiate the sourdough fermentation. The sourdough was fermented at room temperature which varies 19–30°C depending on the season and refreshed every 4 h (Table 1). During breaks in bread production, the sourdough was mixed with flour to lower the water activity and thereby forestall the growth of microorganisms. Even during breaks in production the sourdough was stored at room temperature until the next

fermentation cycle. In order to demonstrate the importance of maintaining stable fermentation parameters, the sourdough (sample D4) was transferred to a temperature regulated kettle with periodic mixing to keep the chemical and microbiological properties of the sourdough stable. From this point on the sourdough is kept at 30°C, fermented for 12 h and renewed with an inoculum of 10 %. During breaks in the production process, the sourdough is removed from the kettle and stored at 3–5°C for up to 36 h. The refrigerated sourdough is then mixed with fresh flour and water in the kettle to initiate the next sourdough fermentation.

Table 1. Industrial sourdough samples obtained from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) with different sourdough propagation parameters. For A_{bakery}, B_{bakery}, and C_{bakery} the time points indicate the time passed from the beginning of a new sourdough propagation. For D_{bakery}, the time points are shown as time elapsed from the collection of the first sample.

Bakery	**A_{bakery}**	**B_{bakery}**	**C_{bakery}**	**D_{bakery}**
Fermentation temperature	32°C	42–44°C	RT (up to 28°C)	RT (19–30°C)
Inoculum size	10 %	6 %	~ 33 %	~ 33 %
Fermentation time	10 h	16 h	~ 4 h	~ 4 h
Dough yield	250	400	~ 200	~ 200
Starter used	Freeze-dried sourdough	Active sourdough	Commercial freeze-dried starter	Commercial freeze-dried starter
Analyzed samples	A0 - freeze-dried sourdough	Bs - 3 years propagated sourdough	C0 - freeze-dried commercial starter	D1 - approx. 30 years propagated sourdough
	A1 - 1.2 months	B0 - active sourdough starter	C1 - 12 months	D2 – 3 months
	A2 - 3.5 months	B1 - 0.25 months	C2 - 21 months	D3 – 5 months
	A3 - 4.5 months	B2 - 1 months	C3 - 28 months	D4 – 16 months (before transfer to 30°C)
	A4 - 8.5 months	B3 - 2.5 months		D5 – 21 months (5 months at 30°C)
		B4 - 6 months		

3.1.2 Laboratory sourdough samples

Laboratory sourdough fermentation was initiated by mixing 150 g of rye flour and 150 g of sterile 0.5 % NaCl solution. This rye flour was the same type used for bread making in most Estonian bakeries (Type 1370, Tartu Mill, Estonia). Three sourdough batches were fermented at 20°C and a further three at 30°C to evaluate the influence of fermentation temperature on the development of microbial communities. After every 24 h of fermentation, each sourdough was renewed with an inoculum of 10 %. In total, 56 fermentation cycles were carried out. The three sourdoughs fermented at 20°C are referred to as 20-I, 20-II, and 20-III while those fermented at 30°C are referred to as 30-I, 30-II, and 30-III.

3.2 Chemical properties of the sourdough samples

3.2.1 pH and titratable acidity

pH and TTA were measured using a DL22 Food and Beverage Analyzer (Mettler-Toledo LLC., USA) at the end of each fermentation cycle prior to renewal. TTA is expressed as volume (in ml) of 0.1 N NaOH used to titrate 10 g of sourdough sample up to a pH of 8.5.

3.2.2 Chemical analysis of organic acids and sugars

High performance liquid chromatography (HPLC) analysis was performed to determine the content of lactic acid, acetic acid, glucose, fructose (together with mannitol), and disaccharides (maltose and sucrose) in the sourdoughs. An HPLC Separations Module Waters 2695 Alliance was used with a Refractive Index Detector 2414 (both from Waters Corporation, USA) and column BioRad HPX-87H Organic Acid Analysis Aminex Ion Exclusion Column 300 × 7.8 mm (Bio-Rad Laboratories Inc., USA). Sulfuric acid solution (0.005 M) was used as an eluent at a flow rate of 0.6 ml min^{-1}. All measurements were conducted at a temperature of 35°C.

3.3 Microbial analysis of sourdoughs using culture-dependent methods

3.3.1 Isolation and enumeration of lactic acid bacteria

Five grams of each sourdough sample was mixed with 45 g of sterile 0.85 % NaCl solution. Decimal dilutions were plated on to either SDB agar (maltose, 2.0 %; yeast extract, 1 %; Tween 80, 0.03 %; trypticase 0.6 %; pH 5.6) or MRS agar (Lab M Ltd, UK) with 100 mg/ml cycloheximide (Sigma-Aldrich, USA). The plates were incubated at the same temperature used for sourdough fermentation, but not lower than 20°C. Incubation was carried out for 48 h under

aerobic or anaerobic conditions (AnaeroGen, Oxoid). Colony forming units (CFU) were counted from the plates with suitable dilutions (usually 20–200 colonies per plate).

3.3.2 Fingerprint typing by Rep-PCR and identification of isolates by Sanger sequencing of the 16S rRNA gene

Genomic bacterial DNA was extracted from isolates using Whatman indicating FTA MiniCards (GE Healthcare Ltd., UK). Rep-PCR with primer $(GTG)_5$ was performed and obtained amplicons were separated using 1 % agarose gel electrophoresis. Each fingerprint type was calculated as the ratio of similar fingerprints to the number of colonies analyzed. One or two representatives from each PCR fingerprint group were subjected to Sanger sequencing. The 16S rRNA gene fragments were amplified using universal primers 27f-YM (Frank *et al*., 2008) and 16R1522 (Weisburg *et al*., 1991) and the sequences obtained were compared with sequences in the GenBank database using the NCBI tool Blastn.

3.4 Microbial analysis of sourdoughs by culture-independent methods

3.4.1 Extraction of whole genomic DNA from sourdough samples

Total genomic DNA extraction was performed using 5 g of sourdough sample that had been homogenized with 45 ml of sterile physiological solution. This suspension was then centrifuged at 4°C for 5 minutes at 1000 g to remove the flour particles. The supernatant was then collected and centrifuged at 4°C for 15 minutes at 5000 g. Each extraction of whole DNA was performed using a GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) according to the manufacturer's protocol (Publications II and III) or using a phenol-chloroform extraction method (Publication I).

3.4.2 Denaturing gradient gel electrophoresis

The V3 region of the 16S rRNA gene was amplified from the whole genomic DNA using the universal primers F357-GC and 518R as described by (Van der Meulen *et al*., 2007). DGGE was carried out using the INGENYphorU system (Ingeny International Bv., Netherlands). Clear visible bands of interest were cut from the gel and incubated in TE buffer (10 mM Tris, pH 7.5, and 1 mM EDTA, pH 8.0) at 37°C for at least 1 h. Eluted DNA was reamplified using primers F357 and 518R and sequenced in a commercial facility (Estonian Biocenter). Sequences were classified to the nearest species using the Blastn algorithm against sequences in the GenBank database (National Center for Biotechnology Information, USA).

3.4.3 Metagenomic sequencing of barcoded 16S rRNA gene amplicons

Universal primers 8F and 357R were used for PCR amplification of the V2–V3 hypervariable regions of 16S rRNA genes (McKenna *et al*., 2008). The amplicon mixtures were pyrosequenced using a 454 GS FLX+ System (Roche 454 Life Sciences, USA). The resulting data were analyzed using the software package MOTHUR, version 1.27.0 (Schloss *et al*., 2009). The closest match at the species level was found for each OTU using the Blastn algorithm against sequences in the GenBank database (National Center for Biotechnology Information, USA) with the parameters of 97 % similarity and 90 % coverage (Publication III) or by comparing the sequences to those within the Greengenes 16S rRNA database at 97 % similarity (Publication II). The relative abundance of OTUs was calculated as the number of sequences for each OTU divided by the total number of bacterial sequences obtained for each sourdough sample. To estimate the beta-diversity, non-metric multidimensional scaling (NMDS) was conducted based on Yue and Clayton distances (Yue and Clayton, 2005) in MOTHUR followed by visualization in R.

3.5 Characterization of carbohydrate fermentation properties and oxygen tolerance of isolated lactic acid bacteria

API 50 CH carbohydrate fermentation tests were performed to evaluate the fermentation capability of isolated LAB species. *Lactobacillus helveticus* CH-1 (Christian Hansen, Denmark), a dairy starter, was used as a reference strain.

For all *L. helveticus* strains, the occurrence and variation of β-galactosidase encoding genes within the *lacLR* intergenic region was determined by amplifying the region with the primers *lacLn* (5'-GACCCTGAATACCTCAGG-3') and *lacRn* (5'-TTCAGCGCATCTCGCTCT-3') (Callanan, Beresford and Ross, 2005).

The influence of oxygen on the growth of *L. helveticus* isolates was evaluated using the BioscreenC system (Oy Growth Curves Ab Ltd, Finland). The selected LAB were grown in MRS broth overnight and decimal dilutions were also performed in MRS broth. 200 µl of dilutions 10^2 and 10^3 were pipetted into BioscreenC wells. Cultivation experiments were carried out at 30°C in both aerobic and anaerobic conditions. To create an oxygen-limited environment, the plate was hermetically sealed in an N_2 atmosphere.

4. RESULTS AND DISCUSSION

The outcome of rye bread making greatly depends on the successful fermentation of sourdough. This intermediate product in the bread making process determines the texture, taste, and aroma of rye bread. The production of metabolites in the dough depends on the composition of the microbial community and applied fermentation parameters. The latter vary significantly among industrial bakeries of different sizes. In Estonia rye bread is manufactured by both small scale bakeries as well as large producers and therefore a wide variety of rye breads are available on the market.

This work focuses on evaluating the influence of sourdough propagation parameters applied in bakeries of different sizes on the microbial and chemical properties of rye sourdough. During the Soviet times, bread production was standardized and sourdough processes were commonly based on pure cultures. Currently, most bakeries have developed their own sourdough and propagate it by daily backslopping (Valjakka, Kerojoki and Katina, 2003). All four bakeries studied kept an in-house rye sourdough that was started from either a mature freeze-dried sourdough (A_{bakery}), a fresh active sourdough from another bakery (B_{bakery}) or from a commercial starter (C_{bakery} and D_{bakery}). In this work, the sourdoughs were monitored to determine the stability of the microbial communities and chemical properties during repeated backslopping.

Rye flour is the main non-sterile raw material and thus is a potential origin for microorganisms to enter industrial sourdoughs. Laboratory spontaneous sourdough propagation was carried out to determine if the rye flour used by all four bakeries can act as a source of sourdough LAB.

4.1 Microbial composition and stability of industrial rye sourdoughs (Publications I and II)

The aim, in Publications I and II, was to determine the effect of sourdough propagation properties on the stability of the microbial communities. Four industrial rye sourdoughs propagated using different schemes were studied and the changes in these sourdoughs over repeated backslopping cycles were tracked. For this, their microbial composition was determined, the stability of the LAB communities they housed was assessed, and their chemical properties were monitored.

4.1.1 Using a well-adapted LAB community to initiate fermentation of a new sourdough

In a large scale industrial bakery, a new rye sourdough cycle was initiated by adding a freeze-dried sourdough to a mixture of rye flour and water. The freeze-dried sourdough had been prepared from a mature sourdough from the same bakery six years prior to this study. Because the freeze-dried starter originated from a sourdough cycle with the same propagation parameters, it was expected

that the LAB community should be well adjusted to those conditions and that a mature rye sourdough suitable for bread making could be obtained within the first few refreshment steps. In a large scale industrial setting this is an important aspect to consider, as it is crucial to obtain high quality rye bread from every production cycle. The microbial stability of the industrial sourdough initiated from freeze-dried starter was monitored for over eight months to determine, whether the LAB strains in the starter are competent enough to remain in the sourdough cycle or if they are replaced by bacteria originating from the raw materials. In order to obtain a comprehensive overview of the community dynamics within sourdough, both culture dependent and culture independent methods were applied to both determine the number of LAB and monitor changes in their microbial composition.

The number of culturable LAB, as determined by plating on MRS, was found to be 10^9 CFU g^{-1} after the first fermentation cycle and remained high throughout the study (Table 2). Higher counts were obtained from anaerobically incubated MRS plates, thus indicating that the sourdough LAB may be sensitive to oxygen. Because this sourdough was fermented in large 800 kg tanks, where the surface area is small compared to the total volume of sourdough, the availability of oxygen within the sourdough is presumably very limited.

The pH and TTA values of the mature sourdough were in the range of 3.5–3.7 and 19–22, respectively (Table 2), which is common for type II rye sourdoughs that typically are more acidic (Hansen, 2004; Weckx *et al*., 2010b). It is important to note that by using a well-adapted LAB community as a starter, the sourdough had a low pH that is characteristic to mature sourdough, already by the end of the first extended fermentation cycle, thereby allowing the baker to produce rye bread. The two main chemical parameters remained stable throughout the entire observation period which generally reflects on the high quality of rye bread produced in this bakery.

The microbial diversity of the sourdough was evaluated using Rep-PCR with the primer $(GTG)_5$ to fingerprint isolates obtained from MRS plates. Selected representatives of each fingerprint type were identified by Sanger sequencing of the 16S rRNA gene. LAB strains from the species *Lactobacillus helveticus*, *L. panis*, *L. pontis,* and *Lactobacillus vaginalis* were found in the freeze-dried starter used to inoculate the new propagation cycle. Most of the isolates obtained from sourdough samples throughout the sampling period, however, belonged to the species *L. helveticus* which is not a typical LAB species in cereal sourdoughs (Hamad *et al*., 1997; Scheirlinck *et al*., 2008) but is commonly used as a starter culture in cheese making. Strains from the species *L. panis*, *L. pontis,* and *L. vaginalis* were also detected in substantial numbers. It was observed, that the culturable portion of *L. helveticus* decreased over time and was replaced by *L. pontis*. This illustrates the adaptation of *L. helveticus* species to the oxygen limited conditions in large scale sourdough production and also its capability to utilize cereal carbohydrates.

Table 2. pH, acidity and bacterial counts on MRS in samples of industrial sourdough. Standard deviations are shown with (±).

Number of renewals	Time from the start of backslopping	pH	TTA (ml 0.1 N NaOH)	Aerobic LAB counts (CFU g^{-1})	Anaerobic LAB counts (CFU g^{-1})
0	0 hours	4.79 ± 0.11	4.98 ± 0.43	$8.91 \pm 0.23 \times 10^4$	$7.08 \pm 0.07 \times 10^5$
1	24 h	3.63 ± 0.06	20.74 ± 0.48	$1.95 \pm 0.02 \times 10^8$	$7.59 \pm 0.04 \times 10^8$
2	43 h	3.53 ± 0.14	21.27 ± 0.15	$4.27 \pm 0.00 \times 10^9$	$1.10 \pm 0.01 \times 10^9$
3	55 h	3.54 ± 0.14	17.66 ± 0.09	$2.40 \pm 0.03 \times 10^8$	$6.46 \pm 0.08 \times 10^8$
4	67 h	3.54 ± 0.12	17.34 ± 0.38	$2.82 \pm 0.03 \times 10^8$	$1.07 \pm 0.01 \times 10^9$
5	77 h	3.66 ± 0.13	15.71 ± 0.27	$1.29 \pm 0.00 \times 10^8$	$5.62 \pm 0.07 \times 10^8$
40	2 weeks	ND	ND	$1.17 \pm 0.05 \times 10^8$	ND
100	5 weeks	3.67 ± 0.06	22.30 ± 0.56	$4.57 \pm 0.02 \times 10^7$	$6.61 \pm 0.05 \times 10^8$
250	14 weeks	3.71 ± 0.01	18.89 ± 0.05	$4.68 \pm 0.06 \times 10^7$	$1.10 \pm 0.01 \times 10^9$
300	16 weeks	3.63 ± 0.02	19.78 ± 0.07	$1.29 \pm 0.00 \times 10^8$	$1.41 \pm 0.01 \times 10^9$
350	18 weeks	3.63 ± 0.04	21.30 ± 0.41	$1.29 \pm 0.00 \times 10^8$	$6.92 \pm 0.05 \times 10^8$
550	34 weeks	3.53 ± 0.02	21.84 ± 0.30	$3.31 \pm 0.03 \times 10^8$	$1.20 \pm 0.00 \times 10^9$
600	35 weeks	3.49 ± 0.02	21.28 ± 0.08	$2.19 \pm 0.02 \times 10^8$	$1.41 \pm 0.02 \times 10^9$

PCR-DGGE analysis was carried out in parallel to plating and Rep-PCR fingerprinting to evaluate the qualitative stability of the microbial community of the sourdough (Figure 4). The results demonstrate that a significant similarity in band patterns occurred over the entire observation period, starting from the very first renewal cycles. Also, it was observed, that the species isolated from freeze-dried sourdough by plating were detected on DGGE. This indicates that all the dominant species survived the freeze-drying process. The LAB species *L. helveticus*, *L. pontis,* and *L. panis* were detected in all sourdough samples throughout the study. No noticeable changes occurred in the microbial community during approximately eight months of daily propagation. The exemplary stability of the microbial community within this sourdough is presumably exhibited as a result of the precisely controlled sourdough fermentation parameters applied in this large-scale industrial production facility.

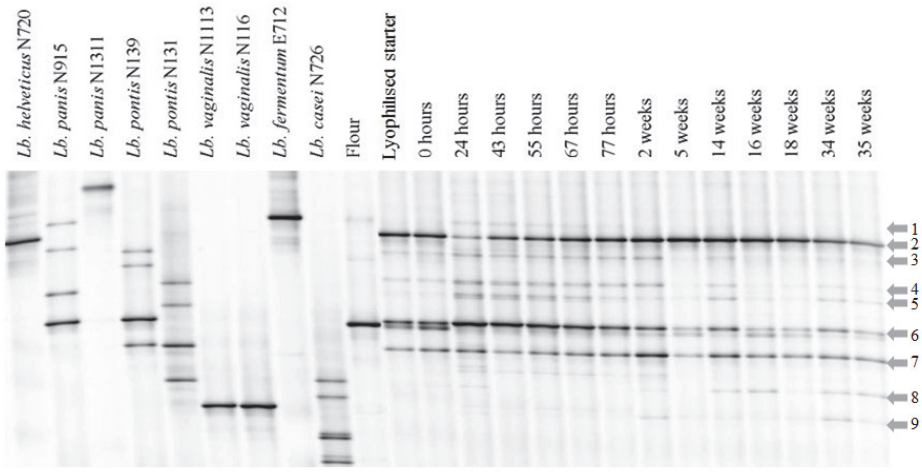

Figure 4. Dynamics of the microbial population in the industrial rye sourdough during over seven months of propagation. Time from the start of the renewal is shown on top. Position of strain-specific and cereal-specific DGGE bands are shown in the left part of the figure. Numbers corresponding to bands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

This study demonstrates that using a previously freeze-dried form of an adapted LAB community to initiate a new sourdough fermentation cycle is a viable alternative to using commercially available starter strains. This approach may also be advantageous because commercial starters may not be competitive under the specific sourdough propagation conditions within individual bakeries. Sub-optimal competitiveness within the bacterial community often results in microflora of the raw materials taking over which can cause serious issues with the quality of the final product. The study further shows that if the LAB used to initiate a sourdough cycle are obtained from a cycle with similar propagation conditions, the microbial community can be stable for a long time, even with daily backslopping using non-sterile raw materials. It is important to note that by applying a well-adapted starter, bread can be successfully made already after the first fermentation cycle, which is of crucial importance in large-scale production.

4.1.2 Microbial composition and stability of four industrial rye sourdoughs

Sourdoughs from four Estonian bakeries were studied to identify the dominant LAB and to monitor the stability of the microbial communities they house. In addition the effect of sourdough propagation parameters on both the microbial and chemical composition of the sourdoughs was evaluated. Two of the sourdoughs studied (C_{bakery} and D_{bakery}) were traditional type I rye sourdoughs

that had a firm structure, were fermented at ambient temperature, and renewed frequently. The other two sourdoughs (A_{bakery} and B_{bakery}) can be classified as type II rye sourdoughs propagated in large-scale bakeries. These sourdoughs had a liquid consistency, were fermented at higher temperatures using temperature-controlled containers, and were renewed less frequently compared with the two type I sourdoughs. The DY of Estonian sourdoughs is very similar to those in Finland (250-300) (Valjakka, Kerojoki and Katina, 2003). Sourdough propagation parameters and time scale of collected samples are shown in Table 1. A comprehensive study on the dynamics of the LAB community within the A_{bakery} sourdough was discussed in the previous section and published in Publication I.

4.1.2.1 Number of lactic acid bacteria, pH and total titratable acidity in industrial rye sourdoughs

The number of culturable LAB was stable in the two type II sourdoughs from A_{bakery} and B_{bakery} and remained within the range of 8.0–9.0 log CFU g^{-1} during most of the sampling regime (Figure 5A); a property characteristic to rye sourdoughs from the Nordic region (Valjakka, Kerojoki and Katina, 2003). In type I sourdoughs from C_{bakery} and D_{bakery}, the LAB counts fluctuated significantly. This is presumably due to the application of inconsistent sourdough propagation parameters, mainly temperature and fermentation time. The ambient temperature fluctuated depending on the season, and fermentation time was not constant during every cycle due to variations in their production schedules. Both factors play an important role in determining the growth of sourdough LAB.

The pH of sourdoughs ranged between 3.50–3.75 in samples obtained from both A_{bakery} and B_{bakery}, which is typical for type II sourdoughs fermented at higher temperatures (Figure 5B). In C_{bakery}, the pH values were constantly above 4.0 which is considered a critical limit above which it is difficult to obtain rye dough with the required rheological properties. Above pH 4.0 the α-amylase activity is insufficiently inhibited and excessive degradation of rye starch can occur (Seibel and Weipert, 2001). In addition, the water binding and solubility of pentosans is problematic. In D_{bakery}, the pH fluctuated depending on the ambient temperature in the bakery. During colder months (sample D1 – collected in February) the pH was considerably higher compared to warmer months, when ambient temperature in the bakery soared close to 30°C (sample D3 – July). This insufficient acidity within the sourdough in winter caused problems with the gas retention of the bread dough, and resulted in rye bread with low volume and poor crumb structure. The pH of the sourdough was not routinely measured in this bakery. Since the production of lactic acid is significantly influenced by temperature and the formation of acetic acid is only marginally temperature dependent, the FQ of the sourdough is shifted towards the higher ratio of acetic acid. This can cause a sharp and acidic taste to the sourdough, which can be misleading when only sensory evaluation is performed to determine the outcome

of sourdough fermentation. The sourdough may appear sufficiently fermented even though the pH is not yet suitable for bread making.

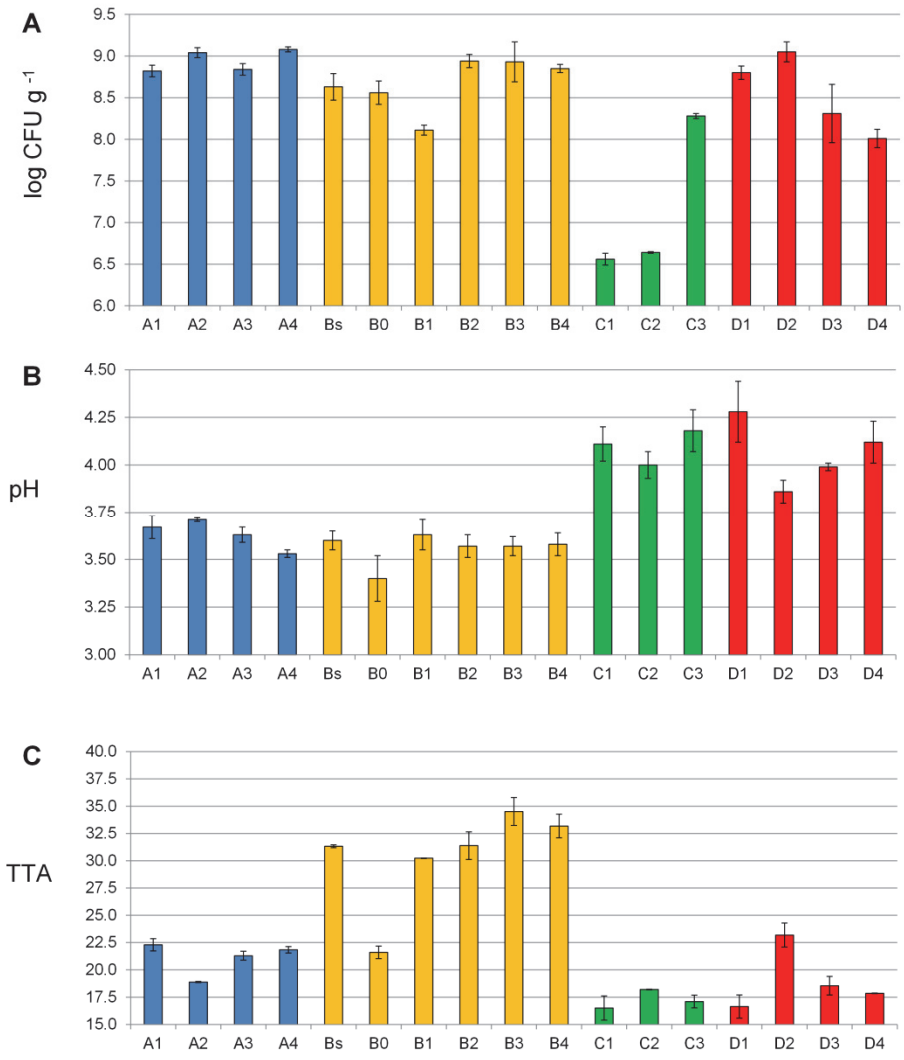

Figure 5. Lactic acid bacteria counts in industrial rye sourdoughs of four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) obtained from MRS plates incubated in anaerobic conditions (A); pH (B) and total titratable acidity (C) of the sourdoughs.

TTA was observed to be highest in B_{bakery}, which can be explained by an unconventionally high fermentation temperature 44°C (Figure 5C). At this temperature large quantities of lactic acid are produced and cause extreme acidification of the sourdough. In both A_{bakery} and B_{bakery}, the ratio of sourdough used in rye bread dough was around 10 %, while in C_{bakery} and D_{bakery} it reached

up to 35 %, depending on the type of bread being produced. Even with the high proportion of sourdough used to make bread dough in the latter two bakeries, the sourdough was unable to assure sufficient acidification, and thereby induced significant problems with dough rheology, gas retention properties, and thus the volume of bread produced. The taste and aroma of the rye breads was also negatively affected. C_{bakery} resorted to adding citric acid to the bread dough in order to increase the acidity rather than allowing the sourdough to ferment longer and/or increasing the fermentation temperature.

4.1.2.2 Chemical properties of industrial rye sourdoughs

Sourdough propagaton parameters applied in the four bakeries studied determine the microbial composition within the sourdough, which, in turn, influences the chemical properties of the sourdough. In addition to pH and TTA measurements, chemical alaysis using HPLC was conducted to detemine the effect of propagation parameters on the utilization of sugars and production of organic acids in industrial rye sourdoughs. It was observed that sourdoughs from A_{bakery} and B_{bakery}, which had strictly controlled sourdough fermentation parameters, showed remarkable stability in their chemical properties compared to sourdoughs from C_{bakery} and D_{bakery}, where the ambient temperature dominated the outcome of the fermentation process (Table 3). Lactic acid production was considerably higher in A_{bakery} and B_{bakery} due to the elevated fermentation temperature because production of lactic acid is favoured at higher temperatures (Spicher and Stephan, 1993). Also, sourdoughs with higher DY have a higher abundance of acids per dry matter. Therefore the differences in DY of sourdoughs from the four bakeries have to be considered.

Sourdoughs from C_{bakery} and D_{bakery} had higher concentrations of fermentable sugars that remained within the sourdough at the end of each fermentation cycle. The fermentation temperature and length of the cycle can both influence the sugar content. In these two bakeries fermentation of the sourdoughs was performed during 4–6 h at a relatively low temperature compared to longer type II sourdough fermentations in the two larger bakeries. During a short fermentation cycle at ambient temperature, the sugars were not completely utilized to produce organic acids. This is common in the fermentation of traditional sourdoughs because maltose is continuously released by flour amylases and is not depleted during the short fermentation cycle (De Vuyst and Neysens, 2005; Minervini *et al*., 2014). Based on the results of both low microbial counts (assessed using LAB) and chemical analysis (high pH and low acid production) it can be suggested that the sourdough fermentation in C_{bakery} was prematurely interrupted. Both of the type I bakeries did not measure the raw materials (rye flour and water) prior to adding them to sourdough during renewal. This aspect may have caused remarkable variations in DY value of sourdough samples obtained from different cycles, which in turn influences the results of chemical analysis that were calculated per dry matter.

Table 3. Concentration of lactic acid and acetic acid in industrial rye sourdough samples obtained from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) with different sourdough propagation parameters. Results are given as µmol g $^{-1}$ in dry matter (DM) to compare sourdoughs with different dough yield.

Sample	Lactic acid, µmol g^{-1}	Acetic acid, µmol g^{-1}	Glucose, µmol g^{-1}	Fructose + mannitol, µmol g^{-1}	Maltose + sucrose, µmol g^{-1}
A1	88.59 ± 0.11	6.99 ± 0.17	0.28 ± 0.00	3.44 ± 0.22	2.19 ± 0.00
A2	77.04 ± 0.00	6.83 ± 0.00	0.67 ± 0.06	3.27 ± 0.22	0.56 ± 0.06
A3	83.59 ± 0.22	6.99 ± 0.17	0.61 ± 0.06	4.00 ± 0.17	0.67 ± 0.09
A4	87.81 ± 0.33	7.49 ± 0.17	0.33 ± 0.00	3.55 ± 0.00	2.10 ± 0.09
Bs	87.26 ± 0.33	16.65 ± 0.00	0.22 ± 0.00	0.61 ± 0.00	2.48 ± 0.03
B0	98.25 ± 0.44	9.99 ± 0.17	0.22 ± 0.06	4.77 ± 0.06	0.50 ± 0.00
B1	104.68 ± 0.44	8.99 ± 0.17	0.28 ± 0.00	4.83 ± 0.28	3.36 ± 0.06
B2	99.58 ± 0.67	12.49 ± 0.33	0.22 ± 0.00	4.05 ± 0.06	3.18 ± 0.06
B4	110.57 ± 0.33	11.66 ± 0.33	0.22 ± 0.11	5.44 ± 0.00	2.95 ± 0.00
C1	19.32 ± 0.00	7.66 ± 0.00	2.39 ± 0.00	8.44 ± 0.11	1.96 ± 0.03
C2	15.76 ± 0.22	6.99 ± 0.50	2.50 ± 0.06	7.60 ± 0.11	2.19 ± 0.09
C3	16.34 ± 0.12	7.11 ± 0.34	3.39 ± 0.05	7.73 ± 0.01	2.08 ± 0.07
D1	31.42 ± 0.22	6.99 ± 0.00	1.94 ± 0.06	1.22 ± 0.06	3.42 ± 0.03
D2	47.29 ± 0.00	14.33 ± 0.00	1.00 ± 0.06	2.05 ± 0.06	3.45 ± 0.03
D5	40.86 ± 0.99	10.49 ± 0.33	0.39 ± 0.00	1.22 ± 0.06	2.86 ± 0.00

4.1.3 Stability of LAB communities within industrial sourdoughs

There is no universal medium to isolate all LAB species let alone strains from sourdough because many species require specific incubation conditions (Vera, Rigobello and Demarigny, 2009). To counter this it was decided to apply DGGE and metagenomic sequencing of barcoded 16S rRNA gene amplicons in parallel to determine the stability of microbial communities and the ratio of LAB species in the industrial sourdough samples. DGGE analysis is able to characterize the qualitative stability of the sourdoughs, as it can detect which species are present, however, it cannot be used to determine the proportion of each species within the bacterial community. The results show that the bacterial

communities within the sourdoughs from A_{bakery} and B_{bakery} were remarkably stable. Species present in either the freeze-dried starter (A0) or fresh sourdough obtained from another bakery and used as an inoculum (B0) remained in the sourdough throughout all propagation cycles (Figure 6).

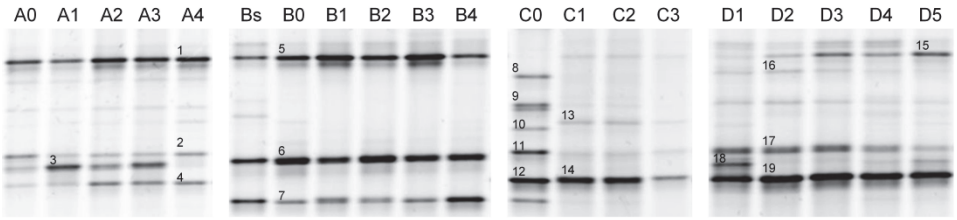

Figure 6. Stability of bacterial communities in industrial rye sourdoughs of four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) determined by DGGE analysis of the 16S rRNA gene amplicons. 1 – Lactobacillus helveticus*; 2* – Lactobacillus panis*; 3 – Cereal chloroplast DNA; 4* – Lactobacillus pontis*; 5* – Lactobacillus amylovorus*; 6 – Cereal chloroplast DNA; 7* – Lactobacillus frumenti*; 8 –10* – Lactobacillus pontis*; 11 – Cereal chloroplast DNA; 12* – Lactobacillus pontis*; 13* – Lactobacillus sanfranciscensis*; 14* – Lactobacillus pontis*; 15* – Lactobacillus helveticus*; 16* – Lactobacillus pontis*; 17 – Cereal chloroplast DNA; 18* – Lactobacillus zymae*; 19* – Lactobacillus pontis.

L. helveticus, *L. panis* and *L. pontis* species were detected in A_{bakery} sourdough throughout the study. B_{bakery} sourdough was dominated by the LAB species *L. amylovorus* and *L. frumenti*, which have been found in type II sourdoughs with higher fermentation temperatures (Müller, Ehrmann and Vogel, 2000; Müller *et al*., 2001). It has been shown, that a high DY combined with higher temperatures favor the growth of homofermentative LAB, in this case *L. amylovorus* (Decock and Cappelle, 2005). These results are in agreement with the diversity of LAB determined by plating.

The DGGE analysis revealed that the freeze-dried commercial starter used in C_{bakery} contained *L. pontis*. However, analyzing the sourdough samples collected over two years showed that *Lactobacillus sanfranciscensis* is a dominant species in all samples. Both of these results do not coincide with the bacterial diversity determined using the culture-dependent approach. It was observed that *L. sanfranciscensis* required an extended incubation time and additional maltose in the medium to be isolated by plating. For this reason *L. sanfranciscensis* was not detected in the first samples (C1 and C2), which were incubated for an insufficient amount of time (48 h). This observation highlights the importance of media selection and incubation conditions to correctly identify sourdough LAB. Because *L. sanfranciscensis* was not detected in the freeze-dried starter, it can be presumed that this species belonged to the in-house microbiota of C_{bakery} and entered the sourdough from either the environment or the raw materials (Scheirlinck *et al*., 2009). It has been shown that, unlike in aseptic laboratory sourdough propagation, the in-house microbiota can play an important role in the development and stability of industrial sourdoughs and also act as a source of LAB (De Vuyst *et al*., 2009; Minervini *et al*., 2012). DGGE analysis of D_{bakery}

sourdough confirmed the results obtained using culture dependent approach. During fermentation at lower ambient temperature, *Lactobacillus zymae*, a species capable of growing at 15°C, was detected and thermophilic *L. helveticus* was not observed in the sourdough. *L. pontis* was prevalent throughout the sampling regime.

In order to obtain information about the relative abundance of LAB in the industrial sourdoughs and overcome the limitations set by the previously described approaches, metagenomic sequencing of the 16S rRNA gene amplicons was performed. This analysis confirmed a conclusion made based on plating and DGGE results that the microbial communities were remarkably stable in both type II sourdoughs of A_{bakery} and B_{bakery}, where sourdough was propagated at strictly controlled conditions (Figure 7).

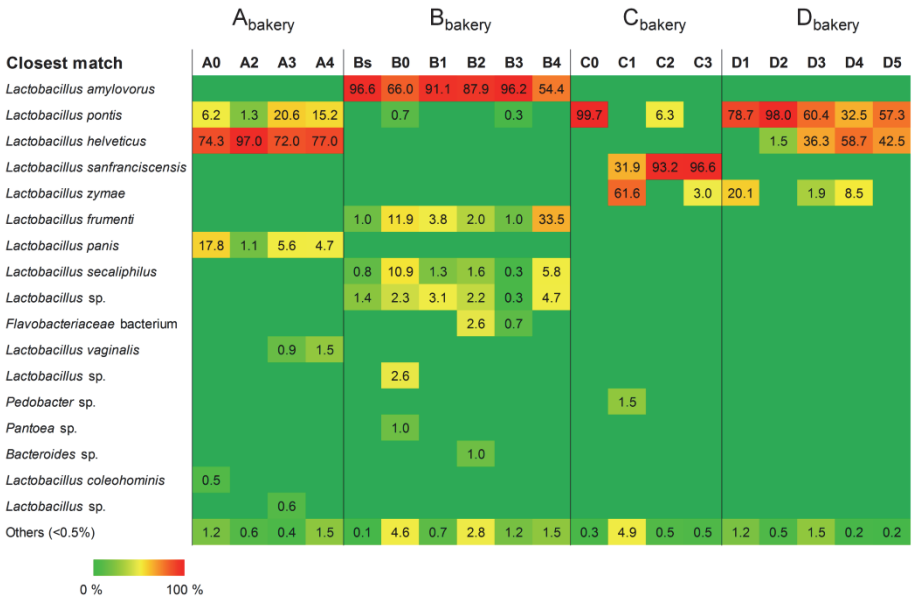

Figure 7. Relative abundance (%) of species in the microbial composition of industrial rye sourdoughs obtained from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) with different sourdough propagation parameters determined by pyrosequencing of barcoded 16S rRNA amplicons, region V2-V3. All OTUs comprising less than 0.5 % are grouped as "Others".

The ratio between LAB species fluctuated somewhat, however, the overall dominant microbial composition remained stable throughout the study. In the two bakeries that fermented type I sourdoughs under ambient conditions, notable changes were seen in the microbial composition during the study. In C_{bakery} it was confirmed that the *L. pontis* used to inoculate the sourdough was unable to prevail in the mature sourdough. The LAB species *L. sanfranciscensis* and *L. zymae* presumably entered the sourdough from raw materials or bakery equipment and were more competitive in the sourdough environment. The

sourdough of D_{bakery} was dominated by *L. pontis* in all samples; however, the ratio of thermophilic *L. helveticus* and cold tolerant *L. zymae* varied significantly with ambient temperature changes induced by seasonal changes.

NMDS was conducted to assess the beta-diversity of the pyrosequenced sourdough samples (Figure 8). This method illustrates the stability of each industrial rye sourdough and also characterizes the similarities between bakeries. The freeze-dried starter from C_{bakery} (C0) clearly clusters together with D_{bakery} samples because they are both rich in *L. pontis*. The remaining C_{bakery} samples contain a high relative abundance of *L. sanfranciscensis*, a species absent in other bakeries, and thus form a separate group. D_{bakery} and A_{bakery} samples share *L. helveticus* and thus cluster in the same quadrant of the NMDS plot. B_{bakery} sourdough is fermented at an unconventionally high temperature and clearly differs from all other sourdough samples. The small distances between samples collected at different time intervals for both A_{bakery} and B_{bakery} show that the microbial communities in these two sourdoughs changed very little over time. This emphasizes the importance of controlling the propagation parameters.

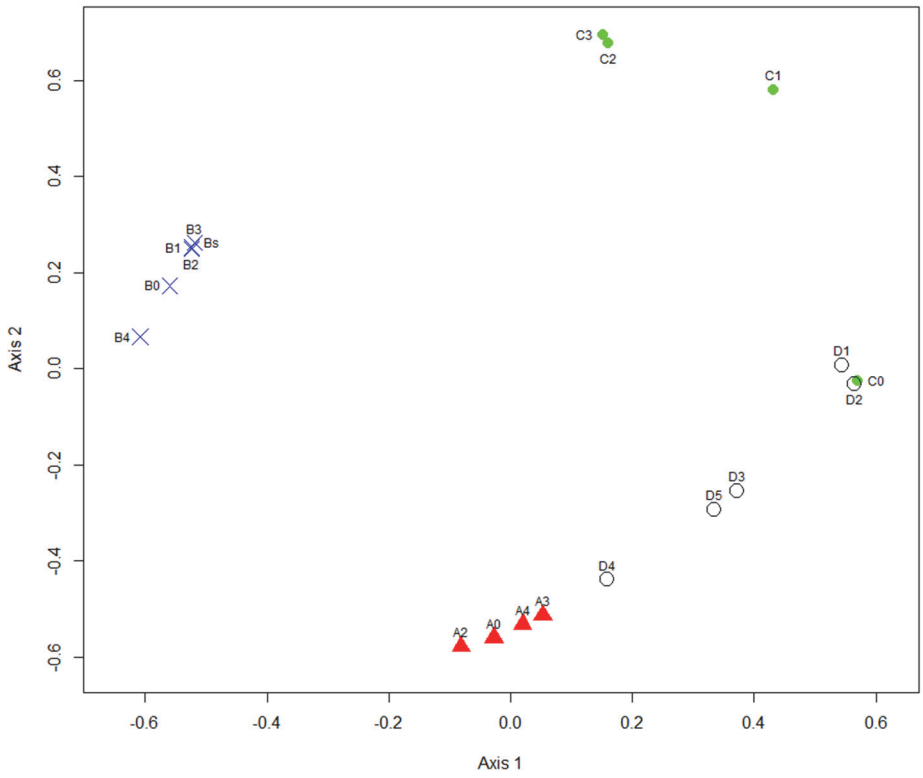

Figure 8. Non-metric multidimensional scaling (NMDS) of industrial sourdough samples in a two dimensional plot with a stress value 0.156 and an R2 value of 0.885.

4.1.4 Influence of new fermentation parameters on the composition of industrial rye sourdough

Changing the fermentation parameters of the sourdough can shift the microbial composition, which, in turn, alters the chemical features of the sourdough (Di Cagno *et al*., 2014). Temperature controlled containers are routinely used in bakeries to standardize the sourdough fermentation process during each production cycle (Valjakka, Kerojoki and Katina, 2003). Also, mixing is typically applied using a programmed schedule to keep the sourdough homogeneous. Both technologies were utilized in A_{bakery} and B_{bakery}. When D_{bakery} sourdough (D4) was taken from room temperature to a 30°C temperature-regulated container and propagated according to a new 12 h cycle for five months (D5), thermophilic *L. helveticus* and *L. pontis* continued to dominate (Figure 9) with the total number of culturable LAB remaining between 8.0–8.5 log CFU g^{-1}. The ratio of LAB and yeast in the sourdough did not change, although it was presumed that higher temperature and longer fermentation time favor the growth of LAB over yeast. The ratio of LAB to yeast remained at around 100:1 in the sourdough (Valjakka, Kerojoki and Katina, 2003; Hansen, 2004).

Before transferring the sourdough to the temperature-controlled system, the pH varied remarkably in samples collected at the end of each fermentation cycle. As a result, D_{bakery} produced bread of variable quality. After temperature control and mixing were adopted, the pH of the sourdough remained at stable low values throughout the remaining observation period (Figure 9). This had a positive effect on bread quality by decreasing the crumb stickiness and improving the gas retention properties. The new 12 h cycle also provided a more flexible production schedule for the bakery because there was no need to renew the sourdough every 4 h.

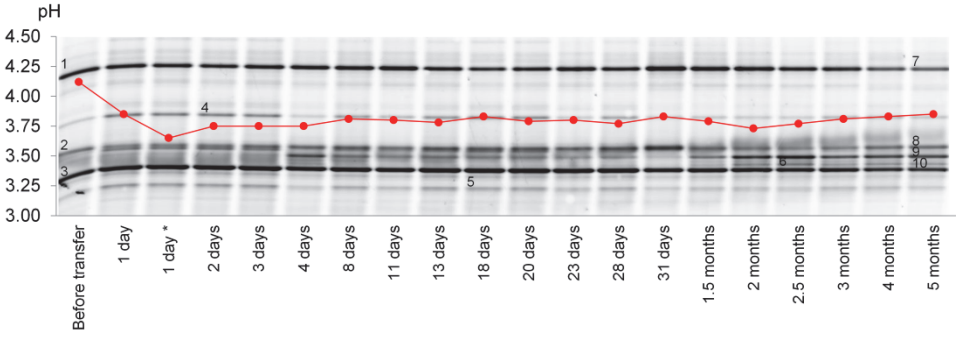

Figure 9. Stability of D_{bakery} sourdough during five months after transferring to temperature regulated kettle. D4 – sourdough before transferring to kettle; D5 – 5 months after transferring to kettle. Time after transfer is indicated above the gel (d – day; m – month), pH of sample is shown in red. 1, 7 – Lactobacillus helveticus*; 2, 8 – Cereal chloroplast DNA; 3, 4, 5, 10* – Lactobacillus pontis*; 6* – Lactobacillus *sp.; 9* – Lactobacillus zymae*. * - sample collected from sourdough after 36 h storage at 5°C.*

4.2 Microbial composition and dynamics of spontaneously started laboratory rye sourdoughs (Publication III)

Flour can strongly affect the microbial diversity of sourdough because it is the only source of nutrients and also carries microorganisms that could potentially become dominant in the sourdough during continuous propagation (De Vuyst *et al*., 2009; Minervini *et al*., 2014). In order to evaluate the potential of rye flour to act as a source of LAB able to compete in a sourdough environment, spontaneous laboratory sourdough fermentation was carried out using the same rye flour used in all four bakeries evaluated in Publication II. During spontaneous laboratory sourdough fermentation, flour is the only non-sterile component and thus the only potential source of microorganisms.

Regardless of the flour type used (wheat or rye), a three-phase evolution of the microbial communities has been described (Van der Meulen *et al*., 2007; Weckx *et al*., 2010b; Minervini *et al*., 2014). Rye sourdoughs spontaneously started using the same rye flour used in the majority of Estonian bakeries were propagated under laboratory conditions for two months to study the evolution of the microbial communities. Sourdoughs were prepared in three parallel fermentations and renewed daily by inoculating a fresh mixture of flour and water with 10 % of the previous batch of fermented sourdough. Because fermentation temperature plays a key role in the formation and further development of LAB communities, the experiment was carried out at two temperatures (20 and 30°C) with three of the sourdoughs propagated at 20°C (20-I, 20-II and 20-III) and a further three at 30°C (30-I, 30-II and 30-III). Prior to this work, several papers had been published studying the evolution of microbial communities in laboratory sourdoughs, however, neither of these experiments lasted over two weeks nor were parallel fermentations performed (Van der Meulen *et al*., 2007; Weckx *et al*., 2010b). Most of the published data concerns wheat sourdoughs with high DY that were propagated while being stirring. In this work, a traditional firm rye sourdough was studied during two months of daily backslopping to determine if the microbial communities within these sourdoughs had reached maturity.

4.2.1 Number of lactic acid bacteria and main chemical properties of laboratory sourdoughs depending on fermentation temperature

Plating on MRS and SDB media was carried out to enumerate the culturable LAB in the sourdoughs (Table 3). The LAB counts were above 9.0 log CFU g^{-1} already after the third cycle and remained high throughout the experiment. Significant differences ($p<0.05$) were detected between the LAB counts obtained from 20°C and 30°C fermentation samples after the third, fifth (only on MRS), 15th (only on MRS) and 56th cycles. This indicates that fermentation temperature did not have a major effect on the number of LAB in the spontaneously started rye sourdoughs. As for the main chemical properties, the average TTA was higher and the pH lower in sourdoughs fermented at 30°C after every cycle.

*Table 4. Enumeration of lactic acid bacteria, together with pH and total titratable acidity (TTA) in spontaneous rye sourdoughs during two months of backslopping; viable counts are given as log CFU g^{-1} obtained on SDB and MRS media. * - statistical difference between counts obtained from 20°C and 30°C ($p<0.05$).*

	Average at 20°C				Average at 30°C			
Cycle	**SDB, log CFU g $^{-1}$**	**MRS, log CFU g $^{-1}$**	**pH**	**TTA, ml 0.1n NaOH /10 g**	**SDB, log CFU g $^{-1}$**	**MRS, log CFU g $^{-1}$**	**pH**	**TTA, ml 0.1n NaOH /10 g**
0	5.30 ± 0.02	5.29 ± 0.02	6.71 ±0.40	2.40 ± 0.58	5.30 ± 0.02	5.31 ± 0.01	6.28 ± 0.06	2.57 ± 0.30
1	8.76 ± 0.06	8.75 ± 0.06	5.95 ± 0.03	5.41 ± 0.36	8.87 ± 0.05	8.84 ± 0.06	5.16 ± 0.29	7.74 ± 0.52
3	9.36 ± 0.02*	9.26 ± 0.02*	4.06 ± 0.11	14.51 ± 0.35	9.13 ± 0.04*	9.08 ± 0.04*	3.83 ± 0.04	16.91 ± 0.79
5	9.38 ± 0.14	9.35 ± 0.07*	3.89 ± 0.13	16.71 ± 0.96	9.34 ± 0.05	9.13 ± 0.05*	3.80 ± 0.04	18.03 ± 1.10
7	9.04 ± 0.13	8.97 ± 0.13	3.95 ± 0.10	16.22 ± 0.17	8.86 ± 0.23	8.99 ± 0.08	3.83 ± 0.05	20.10 ± 1.32
10	9.21 ± 0.16	9.29 ± 0.06	3.93 ± 0.08	17.25 ± 0.75	9.36 ± 0.06	9.35 ± 0.09	3.79 ± 0.06	22.46 ± 0.71
15	9.46 ± 0.09	9.58 ± 0.02*	3.81 ± 0.10	19.15 ± 0.86	9.37 ± 0.07	9.32 ± 0.02*	3.74 ± 0.04	21.67 ± 1.04
21	9.39 ± 0.07	9.31 ± 0.08	3.81 ± 0.10	19.80 ± 0.66	9.26 ± 0.20	9.24 ± 0.20	3.76 ± 0.05	19.90 ± 1.20
28	9.32 ± 0.15	9.28 ± 0.12	3.82 ± 0.13	18.97 ± 0.55	9.25 ± 0.19	9.31 ± 0.12	3.78 ± 0.08	19.04 ± 0.35
42	9.29 ± 0.06	9.31 ± 0.12	3.71 ± 0.14	18.31 ± 0.24	9.24 ± 0.10	9.27 ± 0.08	3.70 ± 0.10	20.09 ± 0.35
56	9.42 ± 0.03*	9.44 ± 0.08*	3.80 ± 0.12	17.10 ± 1.26	9.29 ± 0.02*	9.26 ± 0.05*	3.75 ± 0.05	18.82 ± 0.58

4.2.2 Microbial composition and stability of laboratory sourdoughs depending on fermentation temperature

To determine the evolution of bacterial communities in the sourdoughs during daily backslopping DGGE analysis was performed. It was observed that even after achieving initial maturity, during which *L. plantarum* prevailed in most sourdoughs, further changes occurred in the LAB community and the species was outcompeted by *L. paralimentarius* (Figure 10). In the early stages of propagation the microbial community included a variety of sourdough non-specific species that were able to grow in the not yet acidic conditions (Van der Meulen *et al*., 2007; Weckx *et al*., 2010b). Due to limitations of DGGE analysis identification of these species can be difficult because the weak bands cannot be successfully extracted, reamplified and sequenced. Regardless of the weaknesses of the method DGGE analysis is a fast and relatively simple approach for describing the diversity of microbial communities and for identifying members among the dominant community.

Bar-coded 16S rRNA gene amplicons were sequenced to determine both the evolution of the bacterial communities and identify dominant LAB in mature rye sourdoughs (Figure 11). Differences between the bacterial communities within sourdoughs fermented under the same conditions were observed already after the first fermentation cycle where fermentations at 30°C had a higher prevalence of LAB. During the first few propagation cycles, the number of OTUs detected was higher in sourdoughs fermented at this higher temperature and these sourdoughs established characteristic microbial communities more rapidly than those fermented at 20°C. It is known that only one fermentation cycle is required for *Firmicutes* (including LAB) to gain dominance over Gram negative *Proteobacteria* (Ercolini *et al*., 2013) and that both microbial diversity and number of OTUs reduce over time as sourdough specific LAB begin to dominate (Ercolini *et al*., 2013).

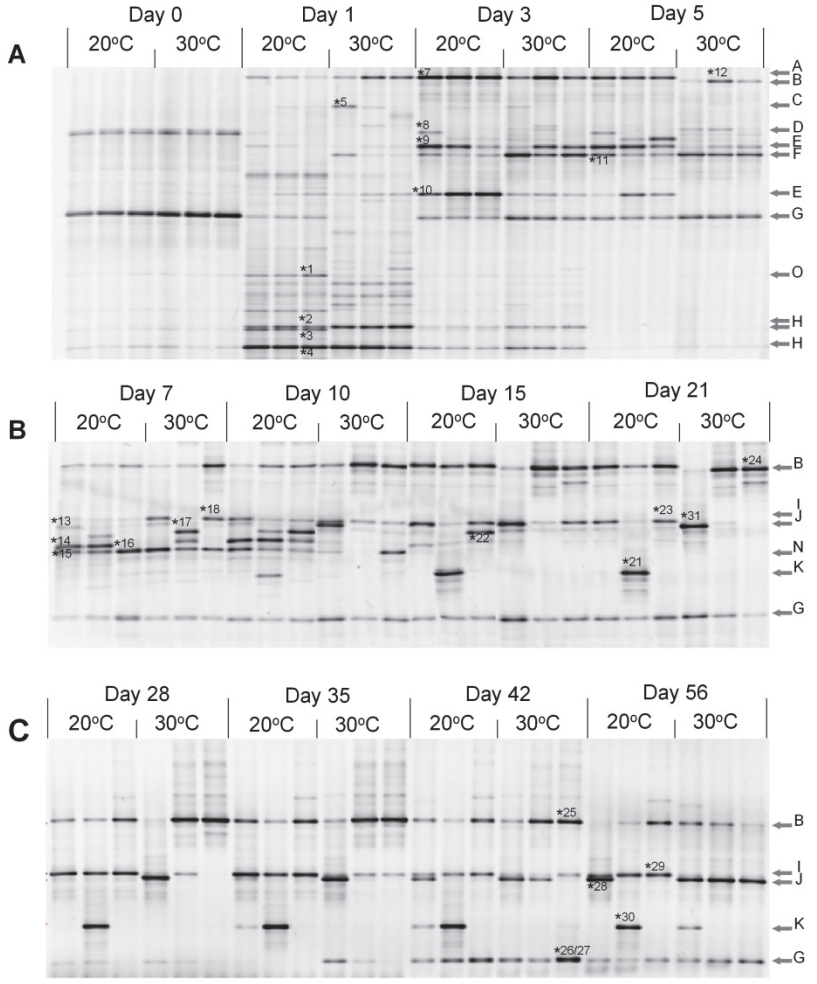

Figure 10. DGGE analysis of six rye sourdoughs propagated at 20°C (20-I, 20-II and 20-III) and 30°C (30-I, 30-II and 30-III) during 56 days. The arrows indicate specific bands for the following groups: (A) Weissella *sp., (B)* Lactobacillus plantarum*, (C)* Enterobacteriaceae*, (D)* Lactobacillus *sp., (E)* Lactobacillus *sp., (F)* Pediococcus *sp., (G) Cereal chloroplast, (H)* Pantoea agglomerans*, (I)* Lactobacillus brevis*, (J)* Lactobacillus paralimentarius*, (K)* Lactobacillus crustorum*, (M)* Lactobacillus curvatus/graminis/sakei*, (N)* Pediococcus pentosaceus*, (O)* Gamma Proteobacteria *species. The bands marked by numbers were cut and sequenced. A. DNA samples from days 0, 1, 3 and 5 of backslopping. B. DNA samples from days 7, 10, 15 and 21 of backslopping. C. DNA samples from days 28, 35, 42 and 56 of backslopping.*

Facultative heterofermentative LAB were prevalent in sourdoughs fermented at 30°C after 56 propagation cycles, while in sourdoughs fermented at 20°C the dominant bacteria consisted of both obligate and facultative heterofermentative LAB. This observation is common in sourdoughs since heterofermentative LAB

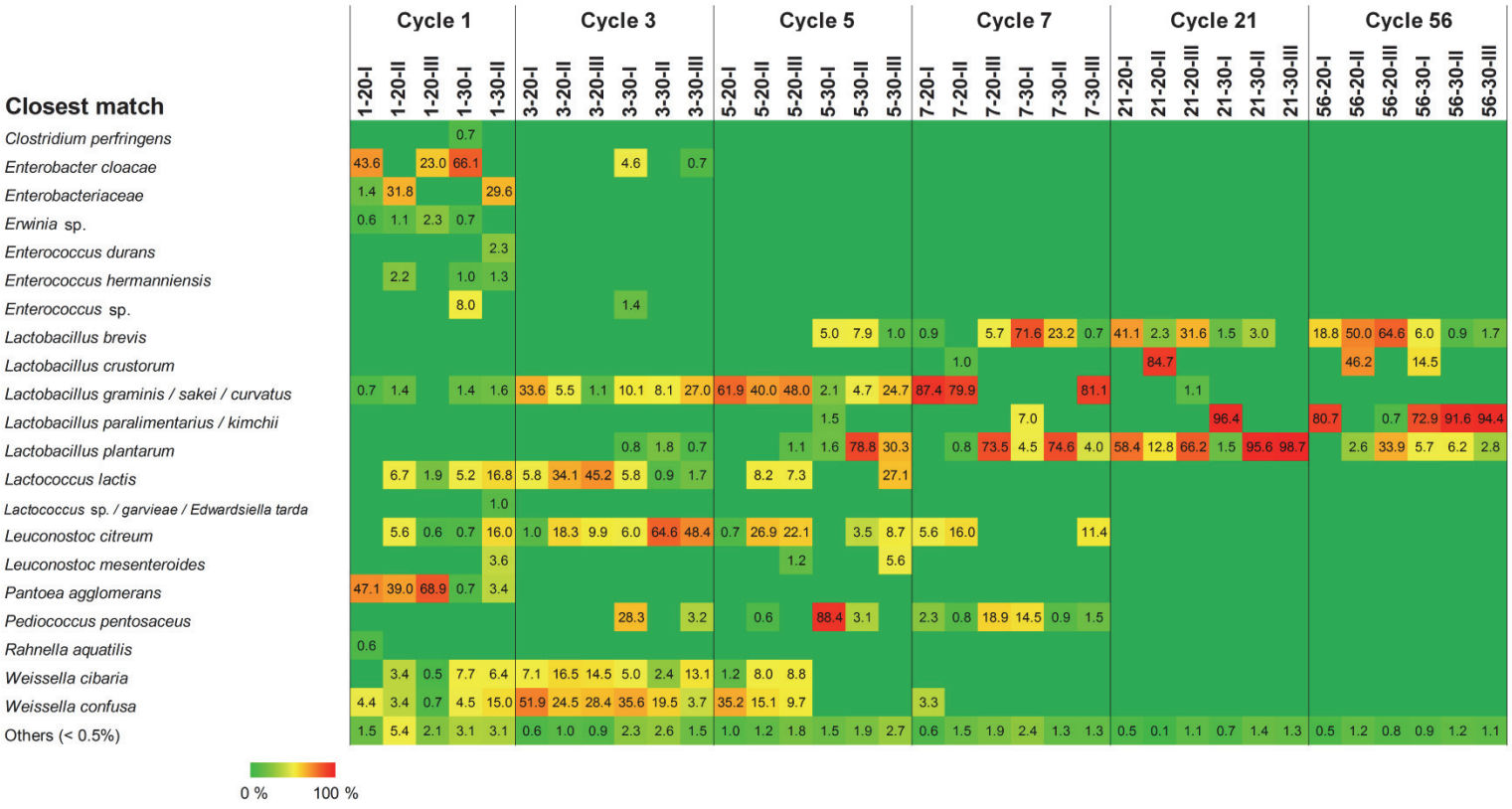

Figure 11. Relative abundance (%) of species in the microbial community of spontaneous laboratory rye sourdoughs during two months of daily backslopping determined by pyrosequencing of barcoded 16S rRNA amplicons, region V2-V3. OTUs with closest matches belonging to same species were grouped. All OTUs comprising less than 0.5 % are grouped as "Others".

have a more flexible metabolism and are capable of using a wider range of cereal carbohydrates. Fermentation temperature altered the relative abundance between LAB species in the community rather than select for different species. During the evolution of bacterial communities in sourdough, *L. plantarum* dominated or co-dominated in most sourdoughs, presumably because of its ability to ferment a wide variety of carbohydrates. However, after reaching initial maturity, *L. plantarum* was replaced by *L. paralimentarius* in sourdoughs fermented at 30°C. At 20°C, the dominant community differed in all three sourdoughs even after 56 propagation cycles. Two LAB species (*L. brevis* in combination with one of *L. plantarum*, *L. crustorum,* or *L. paralimentarius*) co-dominated in these sourdoughs. While the sourdoughs did achieve maturity and followed three stage evolution as described by Van der Meulen *et al*. (2007) and Weckx *et al*. (2010), further succession of LAB species was also observed (Figure 11).

As expected, the rye flour used in this study contained LAB species at a subdominant level, and the majority of these were species that typically do not occur in sourdough communities. The species that dominated in mature sourdoughs may have been present in undetectable concentrations. Further analysis using deep metagenomic sequencing is required to determine the exact microbial composition within rye flour to evaluate the content of sourdough specific LAB. The small number of sourdough competent LAB in the flour combined with the small volume of flour used to prepare the initial sourdoughs may have created an irregular distribution of bacteria between the three parallel sourdoughs propagated at the same temperature. The fate of the microbial communities may have been different if larger quantities of flour had been used. Furthermore, firm low DY sourdoughs have a heterogonous structure which may result in an uneven distribution of LAB. This study revealed that none of the four dominant LAB species found in mature laboratory rye sourdoughs occur in industrial rye sourdoughs propagated using rye flour from the same mill.

The occurrence of and ratio between identified isolates after 56 fermentation cycles was comparable with the relative abundance of species identified using pyrosequencing (Figure 12). The DGGE and pyrosequencing results were comparable despite the fact that different regions of the 16S rRNA gene were analyzed. Pyrosequencing revealed greater bacterial diversity compared to DGGE because this method is more sensitive and can therefore detect species in the sub-dominant population (Ercolini *et al*., 2013; De Vuyst *et al*., 2014). This may be advantageous when studying the early stages of spontaneous fermentation when a wide variety of species are able to grow in the sourdough due to its more neutral acidity.

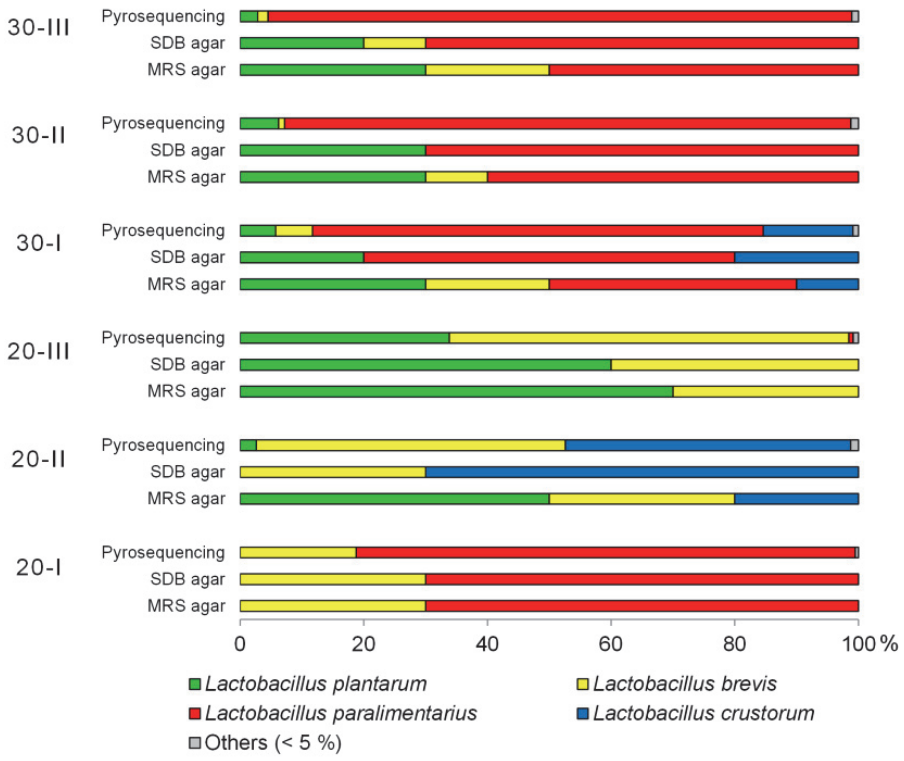

Figure 12. Ratio of species in three sourdoughs fermented at 20°C (20-I, 20-II, 20-III) and three at 30°C (30-I, 30-II, 30-III) after 56 backslopping cycles determined using three methods: plating on MRS media, SDB media, or by pyrosequencing of 16S rRNA gene amplicons.

HTS may not be able to completely resolve differences at the species level, however, it does enables one to describe sub-populations that remain undetected using other approaches (Ercolini *et al*., 2013). In many types of food fermentation, RNA based analysis methods are required to eliminate unwanted amplification from dead cells which can lead to an overestimation of the diversity of metabolically active bacteria. This has not been found to be a problem in the analysis of mature sourdoughs, however, because a similar distribution of OTUs between DNA and RNA samples has been demonstrated (Ercolini *et al*., 2013). Culture independent methods often fail to discriminate between closely related species mainly due to the short sequences involved; however, further development of HTS technologies may resolve this issue.

The most common LAB species isolated from sourdoughs are obligately heterofermentative *L. brevis*, *L. fermentum*, *L. reuteri*, *Lactobacillus rossiae,* and *L. sanfranciscensis*; facultatively heterofermentative *L. alimentarius*, *L. paralimentarius,* and *L. plantarum*, and obligately homofermentative *L.*

amylovorus and *L. delbrueckii* (Huys, Daniel and De Vuyst, 2013; Minervini *et al*., 2014). This work identified several uncommon LAB species within mature rye sourdoughs (Table 5). We report the presence of *L. helveticus* for the first time and *L. amylovorus* for the second time in rye sourdough (Müller *et al*., 2001; De Vuyst *et al*., 2014).

Table 5. Dominating lactic acid bacteria belonging to Lactobacillus *species identified from Estonian industrial and laboratory rye sourdoughs.*

A_{bakery}	B_{bakery}	C_{bakery}	D_{bakery}	Laboratory
Lactobacillus helveticus	*Lactobacillus amylovorus*	*Lactobacillus sanfranciscensis*	*Lactobacillus helveticus*	*Lactobacillus plantarum*
Lactobacillus pontis	*Lactobacillus frumenti*	*Lactobacillus pontis*	*Lactobacillus pontis*	*Lactobacillus brevis*
Lactobacillus panis	*Lactobacillus panis*	*Lactobacillus zymae*	*Lactobacillus zymae*	*Lactobacillus crustorum*
				Lactobacillus paralimentarius

4.3 *Lactobacillus helveticus* – a rare species isolated from industrial rye sourdough (Publications I and IV)

L. helveticus is widely used as a starter in the dairy industry, for example in the production of Swiss cheese (Slattery *et al*., 2010) and is not a common sourdough LAB. *L. helveticus* has been previously isolated from Sudanese sorghum sourdough (Hamad *et al*., 1997) and traditional wheat sourdoughs in Belgium (Scheirlinck *et al*., 2008). However, representatives of *L. helveticus* were found in the dominating population of two industrial rye sourdoughs in A_{bakery} and D_{bakery} that employ different propagation parameters. To study the adapted metabolism of these isolated *L. helveticus* strains, both their carbohydrate fermentation profiles and the influence of oxygen on the growth of *L. helveticus* was described.

Sourdough LAB commonly utilize all four soluble flour carbohydrates: maltose, sucrose, fructose, and glucose (De Vuyst *et al*., 2009). An API 50 CH test was applied to evaluate the carbohydrate fermentation capability of *L. helveticus* isolates in parallel with the dairy starter strain *L. helveticus* CH-1 (Chrtistian Hansen, Denmark). The isolated *L. helveticus* strains (N720, N92 and E96 from A_{bakery} and S64 from D_{bakery}) showed remarkable adaptation to the sourdough conditions and were able to utilize several major carbohydrates available in cereals (Table 6). However, none of the isolates could metabolize lactose, which is the main carbohydrate in milk. These results indicate that the *L.*

helveticus strains isolated from the rye sourdoughs from two bakeries are highly adapted to this cereal environment by being able to ferment several plant carbohydrates while losing the capability to ferment milk.

Table 6. Carbohydrate fermentation test API CH 50 for Lactobacillus helveticus *isolates obtained from industrial sourdoughs (N720, N92, E96 and S64) and commercial dairy starter strain (CH-1) compared to the API CH 50 reference profile.*

Active ingredient in API 50 CH	***L. helveticus* N720**	***L. helveticus* N92**	***L. helveticus* E96**	***L. helveticus* S64**	***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	+	+	+	-	-	-

None of the isolated *L. helveticus* strains were able to metabolize lactose. This striking difference encouraged to investigate the *lacLR* intergenic region (involved in encoding the β-galactosidase enzyme responsible for lactose utilization) in the *L. helveticus* strains isolated from A_{bakery} (Callanan, Beresford and Ross, 2005). No PCR products were observed while using the *lacLn* and *lacRn* primers for any of the three *L. helveticus* strains isolated from A_{bakery} sourdough. However, a 500 bp fragment was obtained for the dairy starter strain *L. helveticus* CH-1 (Callanan, Beresford and Ross, 2005). It can be presumed that genomic rearrangements occurred during adaptation to the cereal environment.

The A_{bakery} sourdough had a high DY and large volume (up to 800 kg), which may limit the amount of oxygen in the sourdough. *L. helveticus* strains isolated from this sourdough are sensitive to oxygen as evidenced by the results from both microcalorimetry (Publication IV) and plating (Publication I) experiments.

To better understand, how the dominant *L. helveticus* strain N92 grows under both aerobic and anaerobic conditions, experiments using the BioscreenC system were carried out. The OD–time growth curves of *L. helveticus* strain N92 was determined in MRS broth at 30°C (Figure 13).

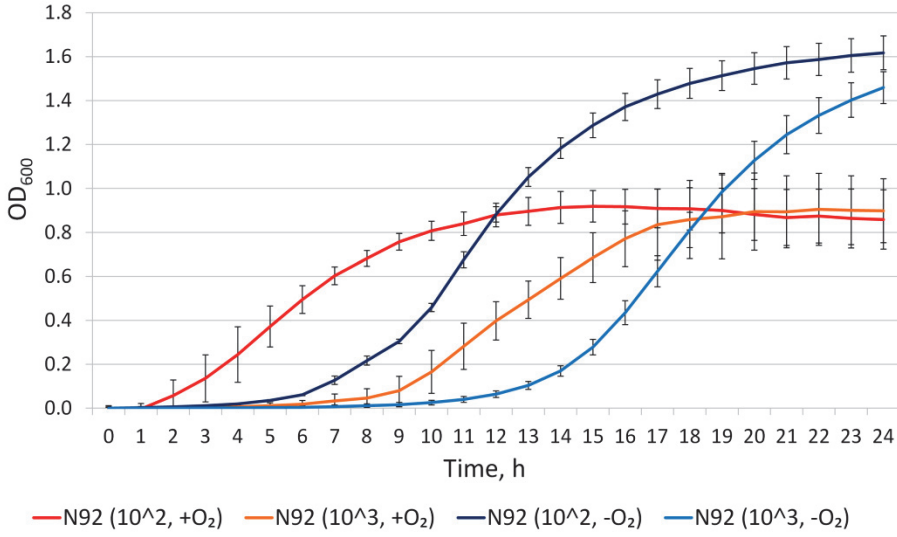

Figure 13. Growth of Lactobacillus helveticus *N92, a dominating LAB isolated from an industrial rye sourdough, in MRS medium at 30°C during 24 hours using different decimal dilutions (10^2 and 10^3). Experiment was carried out in triplicate using the BiocreenC system under both aerobic ($+O_2$) and anaerobic conditions ($-O_2$).*

It was found that in anaerobic conditions, the maximum specific growth rate of the *L. helveticus* N92 strain is approximately two times greater than under aerobic conditions (0.65 h^{-1} versus 0.3 h^{-1}). In addition, a significantly higher OD was achieved under anaerobic conditions during 24 h. This indicates that the *L. helveticus* strain N92 found dominating in the industrial rye sourdough is well adapted to the oxygen deprived environment.

CONCLUSIONS

Microbial communities within traditional sourdoughs can be remarkably stable, provided that the propagation parameters (fermentation temperature and time, inoculum size and dough yield) are kept constant. However, if drastic variations occur in the composition of the raw materials or if fluctuations take place in the propagation conditions, the microbial composition can shift and thereby alter the physical and chemical properties of the sourdough, which often results in undesirable changes in bread quality. This is the first study on the composition and stability of microbial communities of rye sourdoughs used for industrial bread making in Estonia and the Baltic countries and provides valuable knowledge about the populations of LAB within industrial sourdoughs in this region.

The main conclusions that have been drawn based on this work:

I. The stability of microbial communities in industrial rye sourdough depends to a great extent on the stability of the sourdough propagation parameters.

II. A well-adapted community of LAB can be successfully used to inoculate a new sourdough fermentation cycle with similar propagation parameters.

III. *Lactobacillus helveticus* strains isolated from industrial rye sourdough are sensitive to oxygen and their carbohydrate fermentation profiles are adapted to the cereal environment.

IV. Long-term propagated mature sourdough from the spontaneous fermentation of rye flour did not contain any species found to dominate industrial rye sourdoughs made using flour from the same mill.

V. Sourdough LAB have complex nutrient requirements and are often oxygen-sensitive, making them difficult to cultivate using common laboratory media; therefore, culture-independent methods should be additionally employed to study sourdough ecology.

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Summary

Sourdough is a mixture of flour and water that has been fermented with a microbial community consisting of lactic acid bacteria (LAB) and yeast. It is an intermediate product in the bread making process. Fermentation of flour is especially important in rye bread production, since it assures proper structure of the product. The propagation of sourdough is based on regular, often daily, renewal to keep the microorganisms metabolically active. The majority of the resulting sourdough is used for bread making and a small portion is mixed with fresh flour and water and fermented into a new batch of sourdough. Despite the fact that both the raw materials and bakery environment are not sterile, the microbial communities within sourdoughs can remain remarkably stable for many years. Several studies have been published regarding the microbial composition of both industrial and laboratory sourdoughs in many parts of Europe. However, prior to this work there was very little knowledge about the microbial communities within rye sourdoughs used for rye bread production in Eastern Europe. Moreover, most of the published information on this topic is based on phenotypical methods that are insufficient for species level identification.

The aim of this work is to identify lactic acid bacteria in rye sourdoughs used for bread making in four Estonian bakeries that utilize different propagation parameters with the further aim of monitoring the stability of these microbial communities. To determine the availability of LAB in within the rye flour used by all four bakeries, the evolution of microbial communities within spontaneous laboratory rye sourdoughs made from the same flour was investigated during two months of daily backslopping. Both culture dependent and culture independent methods were used for these analyses.

As a result of this work it was found that the composition and stability of the microbial community of each industrial rye sourdough depends on the propagation parameters applied during sourdough fermentation. Bakeries that had strictly controlled sourdough fermentation processes also exhibited remarkable stability in the microbial composition of their sourdoughs, however in bakeries, where fermentation was carried out at ambient temperature, the chemical and microbial properties of the sourdoughs fluctuated and caused problems with the quality of bread produced. In laboratory rye sourdoughs, it was confirmed that microbial communities evolve through a three stage process where species that originate from the flour are gradually replaced by a LAB community consisting of a limited number of well adapted species that are characteristic to the sourdough environment. It was observed that rye sourdough LAB can be difficult to cultivate on laboratory media because they have complex nutrient requirements and can be sensitive to oxygen. Two culture independent methods (DGGE and metagenomic sequencing of 16S rRNA gene amplicons) were applied to identify LAB in sourdoughs and to monitor the stability of those communities during daily propagation. While DGGE provided qualitative information regarding the dynamics of sourdough LAB communities,

metagenomic sequencing of the 16S rRNA gene amplicons allowed us to quantitatively assess the ratio of species in the sourdoughs. This work provides valuable information about the diversity and dynamics of LAB communities within industrial rye sourdoughs and verifies that well controlled propagation parameters reduce quality fluctuations in the end product.

Kokkuvõte

Rukkileivajuuretis on piimhappebakterite ja pärmide toimel käärinud segu jahust ja veest, mis on vajalik traditsioonilise rukkileiva tootmiseks. Kaasaegses leivatööstuses põhineb leivajuuretise kasutamine igapäevasel värskendamisel, mille käigus segatakse leivateost järelejäänud osa juuretisest värske jahu ja veega ning kääritatakse uueks rukkileivajuuretiseks. Selliselt on võimalik hoida juuretist ja selles leiduvaid mikroorganisme metaboolselt aktiivsena ning tagada juuretise käärimisvõime. Kuigi pagaritööstuses kasutatavad toorained ning tootmiskeskkond ei ole steriilsed ning juuretisse satub pidevalt uusi mikroorganisme, võivad juuretise mikroobikooslused püsida muutumatuna aastaid.

Käesoleva ajani on vähe infot igapäevaselt rukkileiva tootmiseks kasutatavate traditsiooniliste rukkileivajuuretiste mikroobikoosluste kohta. Avaldatud on mitmeid teadustöid Kesk- ja Lõuna-Euroopa pagaritööstustes kasutatavate nisuleivajuuretiste omadustest, ent märksa vähem on ilmunud teadusartikleid, mis kirjeldavad Põhja- ja Ida-Euroopas rukkileiva valmistamiseks kasutatavate juuretiste mikrobioloogilist koostist. Käesoleva doktoritöö eesmärgiks oli identifitseerida nelja Eesti leivatööstuse rukkileivajuuretiste koosseisu kuuluvad piimhappebakterid ning jälgida nende bakterikoosluste stabiilsust juuretiste rutiinse värskendamise vältel. Kõik neli pagaritööstust kasutasid leivatootmiseks sama päritolu rukkijahu, ent rakendasid leivajuuretise kääritamiseks erinevaid parameetreid. Et jahu on leivajuuretiste valmistamisel põhiliseks mikroorganismide allikaks, valmistati antud rukkijahust laboritingimustes spontaanse käärimise teel juuretis ning uuriti, kas jahus leidub leivatootmise seisukohalt olulisi piimhappebaktereid.

Doktoritöö tulemusena selgus, et iga tööstusliku rukkileivajuuretise bakterikoosluse stabiilsus sõltus tööstuses juuretise värskendamiseks läbiviidavast protsessist ja kääritamiseks valitud tingimustest. Pagaritööstustes, kus käärimisprotsess oli täpselt kontrollitud, ei esinenud juuretise mikroobikoosluses muutusi ning seeläbi tagati ka leivajuuretise keemiliste omaduste stabiilsus käärimistsüklite lõikes. Nendes pagaritööstustes, kus juuretis kääris toatemperatuuril, esines sõltuvalt aastaajast kõikumisi nii juuretise mikrobioloogilises kui keemilises koostises, mis põhjustas probleeme rukkileiva kvaliteediga. Laboritingimustes kääritatud spontaansetes juuretistes toimus bakterikoosluste areng varem kirjanduses kirjeldatud kolmefaasilise evolutsiooni põhimõtte järgi. Selgus, et kahe kuu jooksul igapäevaselt värskendatud rukkileivajuuretises ei leidunud samu piimhappebakteriliike, mis domineerisid nelja uuritud pagaritööstuse leivajuuretistes.

Antud töös näidati, et rukkileivajuuretistesse kuuluvaid piimhappebaktereid on keeruline laboritingimustes kunstlikel söötmetel kultiveerida, kuna nad vajavad kasvuks erinevaid toitaineid ning võivad olla hapnikutundlikud. Seetõttu rakendati juuretiste uurimiseks kaht kultuursõltumatut analüüsimeetodit (16S rRNA amplikonide DGGE analüüs ja metagenoomne sekveneerimine), et identifitseerida kõik bakteriliigid ning jälgida mikroobikoosluste muutlikkust

juuretiste värskendamise vältel. DGGE analüüs võimaldas küll määrata juuretiste liigilise koostise, ent meetodi puuduste tõttu ei andnud infot nende liikide suhtelise sisalduse kohta mikroobikoosluses. Piimhappebakterite liigiline proportsioon ning alampopulatsiooni kuuluvate bakteriliikide olemasolu tuvastati kogu juuretiseproovist eraldatud DNA metagenoomse sekveneerimise tulemusena. Väljakülvide, DGGE ja kõrge läbilaskevõimega sekveneerimismeetodi kombineerimine võimaldas saada põhjaliku ülevaate Eesti pagaritööstustes rukkileiva valmistamiseks kasutatavate leivajuuretiste mikroobikooslustest. Antud töö tulemused kinnitavad, et kvaliteetse rukkileiva tootmiseks on oluline leivajuuretise käärimisprotsessi täpselt kontrollida ning seeläbi vältida muutusi juuretise mikroobikooslustes ja keemilistes omadustes.

Curriculum vitae

1. Personal data

Name	Ene Viiard
Date of birth	14.05.1985
Place of birth	Tallinn, Estonia
Citizenship	Estonian

2. Contact information

E-mail	ene@tftak.eu
Address	Akadeemia tee 15A, Tallinn 12618, Estonia
Contact phone	+372 505 4748

3. Education

2009 – present	Tallinn University of Technology, Faculty of Chemical and Materials Technology, doctoral studies
2007 – 2009	Tallinn University of Technology, Faculty of Chemical and Materials Technology, MSc *cum laude* in Food Engineering and Product Development; master thesis "*Diversity and stability of lactic acid bacteria in rye sourdough during continuous propagation*"
2004 – 2007	Tallinn University of Technology, Faculty of Chemical and Materials Technology, BSc in Food Engineering and Product Development
1992 – 2004	Keila Gymnasium

4. Language competence

Estonian	Native speaker
English	Proficient User (C2)
Finnish	Independent User (B1)
Russian	Basic User (A1)

5. Professional employment

2008 – present	Competence Center of Food and Fermentation Technologies, researcher; Rye sourdough stability

6. Special courses

2013	One week workshop "Microbial Genomics and Metagenomics", Department of Energy Joint Genome Institute, Walnut Creek, CA, USA
2010	Two weeks PhD course "Molecular Food Microbiology", University of Copenhagen, Denmark

7. Supervised theses

Currently supervising two master students (Käty Metusala and Anna Badulina) and one bachelor student (Kaisa Kuus).

2013	Ann Saarepuu, Bachelor's Degree, "*Sensory evaluation and texture profile analysis of bread*", Department of Food Processing, Tallinn University of Technology
2013	Viivika Lai, Bachelor's Degree, "*Dynamics of microbial community of rye sourdough upon changes in technological parameters*", Department of Gene Technology, Tallinn University of Technology
2012	Kadri Sepp, Bachelor's Degree *cum laude*, "*Optimization of a traditional industrial rye bread recipe*", Department of Food Processing, Tallinn University of Technology
2010	Marianna Bessmeltseva, Master's Degree, "*Technological properties of lactic acid bacteria isolated from industrial rye sourdough*", Department of Food Processing, Tallinn University of Technology
2009	Svetlana Morozova, Master's Degree, "*Characterization of an industrial rye sourdough*", Department of Food Processing, Tallinn University of Technology

Elulookirjeldus

1. Isikuandmed

Nimi	Ene Viiard
Sünniaeg	14.05.1985
Sünnikoht	Tallinn, Eesti
Kodakondsus	Eesti

2. Kontaktandmed

E-post	ene@tftak.eu
Aadress	Akadeemia tee 15A, Tallinn 12618, Eesti
Telefon	+372 505 4748

3. Hariduskäik

2009 – …	Tallinna Tehnikaülikool, Keemia- ja materjalitehnoloogia doktoriõpe
2007 – 2009	Tallinna Tehnikaülikool, Toidutehnika ja tootearenduse magistriõpe (MSc, *cum laude*)
2004 – 2007	Tallinna Tehnikaülikool, Toidutehnika ja tootearenduse bakalaureuseõpe (BSc)
1992 – 2004	Keila Gümnaasium

4. Keelteoskus

Eesti keel	Emakeel
Inglise keel	Kõrgtase (C2)
Soome keel	Kesktase (B1)
Vene keel	Algtase (A1)

5. Teenistuskäik

2008 – …	Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus, Teadur

6. Täiendõpe

2013 (1 nädal)	Kursus "Microbial Genomics and Metagenomics", Department of Energy Joint Genome Institute, Walnut Creek, CA, USA
2010 (2 nädalat)	PhD kursus "Molecular Food Microbiology", Kopenhaageni Ülikool, Taani

7. Juhendatud lõputööd

Hetkel juhendamisel kaks magistritööd (Käty Metusala ja Anna Badulina) ja üks bakalaureusetöö (Kaisa Kuus).

2013	Ann Saarepuu, bakalaureusekraad, "*Leiva sensoorne ja tekstuurianalüüs*", Toiduainete instituut, Tallinna Tehnikaülikool
2013	Viivika Lai, bakalaureusekraad, "*Tööstusliku rukkijuuretise mikroobikoosluse muutlikkus uue tehnoloogilise skeemi rakendamisel*", Geenitehnoloogia instituut, Tallinna Tehnikaülikool
2012	Kadri Sepp, bakalaureusekraad *cum laude*, "*Traditsioonilise rukkileiva tööstusliku retsepti optimeerimine*", Toiduainete instituut, Tallinna Tehnikaülikool
2010	Marianna Bessmeltseva, magistrikraad, "*Tööstuslikust haputaignast isoleeritud piimhappebakterite tehnoloogiliste omaduste iseloomustamine*", Toiduainete instituut, Tallinna Tehnikaülikool
2009	Svetlana Morozova, magistrikraad, "*Tööstusliku rukkihaputaigna juuretise mikrobioloogilise koosluse iseloomustamine*", Toiduainete instituut, Tallinna Tehnikaülikool

Publication I

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ORIGINAL ARTICLE

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

E. Viiard[1,2], A. Mihhalevski[1,2], T. Rühka[3], T. Paalme[1,2] and I. Sarand[1,2,4]

1 Competence Center of Food and Fermentation Technologies, Tallinn, Estonia
2 Department of Food Processing, Tallinn University of Technology, Tallinn, Estonia
3 Leibur Ltd., Tallinn, Estonia
4 Department of Gene Technology, Tallinn University of Technology, Tallinn, Estonia

Keywords
back-slopping, industrial sourdough, lactic acid bacteria, *Lactobacillus helveticus*, rye sourdough.

Correspondence
Inga Sarand, Competence Center of Food and Fermentation Technologies, Akadeemia tee 15 A, 12618, Tallinn, Estonia.
E-mail: 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 7 months.
Methods and Results: The sourdough was started from a 6-year-old freeze-dried sourdough originating from the same bakery. A unique microbial consortium consisting mainly of 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 *Lact. 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 7 months. *Lactobacillus 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 the study: The use of an established and adapted microbial consortium as a starter is a good alternative to commercial starter strains.

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 nonaseptic conditions (De Vuyst and Neysens 2005). According to the study by Van der Meulen *et al.* (2007), it may take as short as 10 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 6 years ago. The microbial community was monitored during 7 months of daily propagation using both culture-dependent and culture-independent approaches.

Materials and methods

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 the inoculation of the new sourdough was prepared from the sourdough of the same bakery 6 years before. It was stored at −20°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 the ratio of 10 : 36 : 54 and incubating for 24 h at 32°C. The following four back-slopping renewals were performed in the same ratio after 19, 12, 12 and 10 h of fermentation. Routine industrial back-slopping was carried out with the same mother sponge, rye flour and water ratio of 10 : 36 : 54 (sourdough yield 250 kg dough per 100 kg flour) and fermentation at 32°C for 10 h. Samples for chemical and microbiological analysis were collected at the end of fermentations.

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., Columbus, OH, 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.

Enumeration of lactic acid bacteria

The number of colony forming LAB 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 min. Series of decimal dilutions were plated on MRS agar (LabM, Lancashire, UK) in triplicate. Plates were incubated at 30°C in both aerobic and anaerobic conditions for 48 h. The BD GasPak EZ System (Becton Dickinson Microbiology Systems, Franklin Lakes, NJ, USA) was used to create anaerobic environment.

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 denaturing gradient gel electrophoresis (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 mmol l^{-1} Tris–HCl (pH 8·0), 50 mmol l^{-1} EDTA] and resuspended in 300 μl STET buffer [8% sucrose, 5% Triton X–100, 50 mmol l^{-1} Tris–HCl (pH 8·0) and 50 mmol l^{-1} EDTA]. A volume of 75 μl of TES lysis buffer containing 1170 U ml^{-1} mutanolysin and 100 mg ml^{-1} lysozyme and 100 μl of proteinase K (2·5 mg ml^{-1}) (all enzymes from Sigma-Aldrich Co. LLC., St Louis, MO, USA) was added, and samples were incubated at 37°C for 1 h. After incubation, 40 μl of preheated (37°C) 20% SDS in TE buffer and a pinch of glass beads (diameter 150–212 μm) were added. Samples were mixed on vortex for 1 min and incubated at 37°C for 10 min, followed by incubation at 65°C for 10 min. 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 mol l^{-1} sodium acetate and two 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 mmol l^{-1} Tris–Cl, 1 mmol l^{-1} EDTA, pH 8·0; AppliChem GmbH, Darmstadt, Germany).

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 analysed by Rep-PCR. Twenty 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 was carried out in 25-μl volume using the following cycle: preliminary denaturation 6 min at 95°C; amplification in 30 cycles: denaturation 94°C for 1 min, annealing 1 min at 40°C, extension 8 min at 65°C and final extension at 65°C for 16 min. Share of each LAB fingerprint type among aerobically and anaerobically selected isolates was calculated as the ratio of similar fingerprints to the number of analysed colonies.

16S 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, Vilnius, Lithuania) and set up for sequencing PCR using BigDye Terminator v3.1 Cycle Sequencing kit as described by manufacturer (Applied Biosystems, Foster City, CA, 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).

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., Tartu, 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 mol l^{-1} urea) was used. Electrophoresis was performed with the INGENYphorU system (Ingeny BV International, goes the Netherlands) at constant voltage 75 V and temperature 60°C for 18 h. The gels were stained with ethidium bromide and photographed with the ImageQuant 400 system (GE Healthcare, Little Chalfont, UK). 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) combined with the TransformAid Bacterial Transformation kit (Fermentas). Cloned DNA fragments were sequenced using the M13 primer.

Determination of carbohydrate fermentation profiles and proteolytic activity of *Lactobacillus helveticus*

Metabolic profiles of *Lact. 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, Marcy l'Etoile, France). Skim milk agar was used to assess the ability of the bacteria to hydrolyse casein (Marcy and Pruett 2001). The test was performed at both 30 and 37°C in aerobic and anaerobic conditions. *Lactobacillus helveticus* CH-1 (Chr. Hansen, Hørsholm, Denmark) was used as a reference strain.

Results

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 with the freshly fermented sourdough (Table 1). This may indicate that freeze-drying and storage during 6 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 of 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 7 months, the number of LAB stayed around 10^9 CFU g^{-1} (Table 1). Colony forming numbers were generally 10 times lower on plates incubated in aerobic conditions (Table 1).

Acidity of the renewed sourdough quickly increased to 20 ml 0·1 N NaOH per 10 g sourdough during the first 24 h of incubation (Table 1). The acidity slightly decreased after third- to fifth-back-slopping step, presumably due to decreased fermentation time. After 5 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).

Table 1 pH, acidity and bacterial counts in the 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 per 10 g sourdough	Aerobic LAB counts CFU g^{-1}	Anaerobic LAB counts CFU g^{-1}
0	0 h	4·79 ± 0·11	4·98 ± 0·43	8·91 ± 0·23 × 10^4	7·08 ± 0·07 × 10^5
1	24 h	3·63 ± 0·06	20·74 ± 0·48	1·95 ± 0·02 × 10^8	7·59 ± 0·04 × 10^8
2	43 h	3·53 ± 0·14	21·27 ± 0·15	4·27 ± 0·00 × 10^9	1·10 ± 0·01 × 10^9
3	55 h	3·54 ± 0·14	17·66 ± 0·09	2·40 ± 0·03 × 10^8	6·46 ± 0·08 × 10^8
4	67 h	3·54 ± 0·12	17·34 ± 0·38	2·82 ± 0·03 × 10^8	1·07 ± 0·01 × 10^9
5	77 h	3·66 ± 0·13	15·71 ± 0·27	1·29 ± 0·00 × 10^8	5·62 ± 0·07 × 10^8
40*	2 weeks	ND	ND	1·17 ± 0·05 × 10^8	ND
100*	5 weeks	3·67 ± 0·06	22·30 ± 0·56	4·57 ± 0·02 × 10^7	6·61 ± 0·05 × 10^8
250*	14 weeks	3·71 ± 0·01	18·89 ± 0·05	4·68 ± 0·06 × 10^7	1·10 ± 0·01 × 10^9
300*	16 weeks	3·63 ± 0·02	19·78 ± 0·07	1·29 ± 0·00 × 10^8	1·41 ± 0·01 × 10^9
350*	18 weeks	3·63 ± 0·04	21·30 ± 0·41	1·29 ± 0·00 × 10^8	6·92 ± 0·05 × 10^8
550*	34 weeks	3·53 ± 0·02	21·84 ± 0·30	3·31 ± 0·03 × 10^8	1·20 ± 0·00 × 10^9
600*	35 weeks	3·49 ± 0·02	21·28 ± 0·08	2·19 ± 0·02 × 10^8	1·41 ± 0·02 × 10^9

*Approximate numbers.
ND, not determined.

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 analysed 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 *Lact. helveticus*, *Lact. panis*, *Lactobacillus pontis* and *Lactobacillus vaginalis* species were identified among isolates obtained from the lyophilized starter. Most of the randomly picked isolates belonged to species *Lact. helveticus* irrespective of incubation conditions (Table 2). Species identified from the lyophilized starter were represented by one (*Lact. helveticus* N92 and *Lact. vaginalis* N1113) or two (*Lact. panis* N915, N1311 and *Lact. pontis* N139, N131) fingerprint types (Fig. 1, Table S2).

Representatives of *Lact. helveticus* species were the dominating aerobic isolates obtained from industrial rye sourdough samples during the 7-month-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 *Lact. vaginalis*, *Lact. fermentum* or *Lactobacillus paralimentarius*.

Lactobacillus 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 *Lact. pontis* and *Lact. panis* (Table 2). Representatives of *Lact. vaginalis* and *Lactobacillus casei* were sporadically detected among the subdominant population.

Three fingerprint patterns were detected for *Lact. helveticus* species (N720, N92 and E96), two fingerprint patterns for *Lact. panis* (N915 and N1311), *Lact. pontis* (N139 and N131) and *Lact. vaginalis* (N1113 and N116) species, and one fingerprint pattern for *Lact. casei* (N726), *Lact. fermentum* (E112) and *Lact. paralimentarius* (E712) species (Fig. 1). *Lactobacillus helveticus* bacteria with N92 and E96 Rep-PCR profiles were isolated in both aerobic and anaerobic conditions, and *Lact. helveticus* N720 fingerprint type was from a single isolate from sourdough sample obtained 5 weeks after the start of the new sourdough cycle. Only *Lact. helveticus* N92 bacteria were isolated from the lyophilized starter, and they were also the dominating type among all *Lact. helveticus* isolates in the industrial sourdough during 7 months of propagation (Table S2).

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

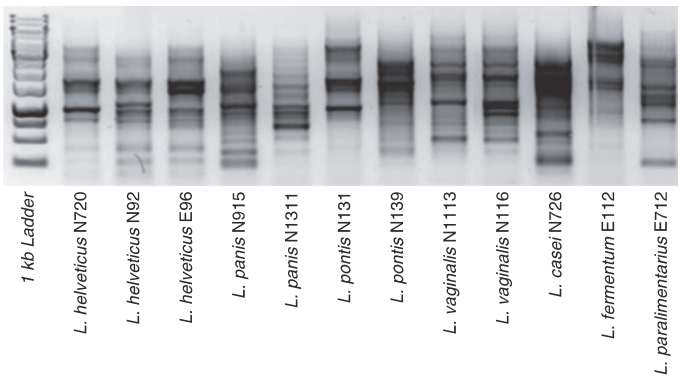

Figure 1 Rep-PCR profile of fingerprint types isolated from the industrial sourdough samples. A 1-kb DNA ladder is used as reference in the first lane.

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 analysed colonies

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

characterize changes in the microbial consortium of sourdough. Preliminary PCR-DGGE analysis of the isolated LAB revealed that bacteria identified as *Lact. helveticus*, *Lact. vaginalis* and *Lact. fermentum* species produced a single separately migrating band on the gel. Bacteria belonging to *Lact. panis*, *Lact. pontis* and *Lact. 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 7-month period. Bands with positions corresponding to the dominating LAB species *Lact. helveticus*, *Lact. pontis* and *Lact. 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 7 months of propagation. Only the detectable appearance of *Lact. vaginalis* and decrease in *Lact. panis* as a result of daily propagation could be suggested based on the DGGE analysis of the industrial sourdough (Fig. 2). Identity of all bands was additionally confirmed by sequencing analysis.

Metabolic profiles of *Lactobacillus helveticus* isolates

16S rRNA gene sequencing and PCR-DGGE analysis determined *Lact. helveticus* as dominating species in the lyophilized starter and industrial sourdough. However, *Lact. 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 *Lact. helveticus* isolates in parallel to *Lact. helveticus* CH-1 strain used in the dairy industry.

Three *Lact. helveticus* strains (*Lact. helveticus* N720, *Lact. helveticus* N92 and *Lact. 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).

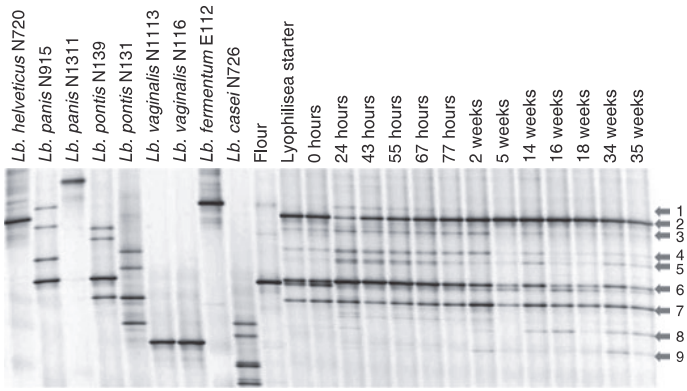

Figure 2 Dynamics of the microbial population in the industrial rye sourdough during 7 months of propagation. Time from the start of the renewal is shown on top. Position of strain-specific and cereal-specific DGGE bands is shown in the left part of the figure. Numbers corresponding to bands identified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

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

Carbohydrate in API 50CH	*Lact. helveticus N720*	*Lact. helveticus N92*	*Lact. helveticus E96*	*Lact. helveticus CH-1*	*Lact. 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	+	+	+†	–	–

*Metabolic profile of *Lact. helveticus* in API test identification table.
†Positive after 72-h incubation.

Maltose and mannose were fermented by *Lact. helveticus* E96 and N720, but not by the N92 strain representing dominating fingerprinting group of the sourdough. The *Lact. helveticus* N720 also fermented galactose and *N*-acetyl glucosamine. None of the three *Lact. helveticus* isolated from the rye sourdough could ferment lactose, contrary to *Lact. helveticus* CH-1 strain, which fermented lactose, mannose, fructose, glucose and galactose.

We determined the proteolytic activity of *Lact. helveticus* N92, E96 and N720 strains and the reference dairy strain *Lact. 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 hydrolyse casein (Table 4). None of the strains had trypsin or α-chymotrypsin activity. The activity of specific arylamidases that catalyse the hydrolysis of N-terminal amino acid from peptides, amides or arylamides differed among the tested strains (Table 4).

Discussion

Lactobacillus helveticus was the dominant LAB species in the lyophilized starter and industrial sourdough samples. Unlike *Lact. panis* (Wiese *et al.* 1996) and *Lact. pontis* (Vogel *et al.* 1994) also detected among dominant LAB population of the studied sourdough, *Lact. helveticus* is not a common sourdough LAB. It is often found in fermented milk products and is used as a cheese starter culture (Slattery *et al.* 2010; Broadbent *et al.* 2011). So far, *Lact. 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 *Lact. helveticus* isolated from sourdough in our work significantly differed from those determined for type strain and the dairy starter.

Table 4 Proteolytic activity of *Lactobacillus helveticus isolates*. Weak reaction is indicated as 'v'

Enzyme tested	*Lact. helveticus* N720	*Lact. helveticus* N92	*Lact. helveticus* E96	*Lact. 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.

BLAST analysis of partial 16S rRNA gene sequences of isolates N92, E96 and N720 (GenBank ID: HM641233, HM623785 and HM641232, respectively) first determined *Lactobacillus suntoryeus* as the closest match (data not shown). *Lactobacillus suntoryeus* is a recently identified species (Cachat and Priest 2005), which afterwards was claimed to be a later synonym of *Lact. helveticus* (Naser *et al.* 2006). Representatives of *Lact. 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 *Lact. helveticus* belong to thermophilic homofermentative lactobacilli (Cachat and Priest 2005).

In spite of the high similarity of *Lact. helveticus* and *Lact. 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). *Lactobacillus 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 *Lact. 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, *Lact. helveticus* strains isolated in our work were able to utilize cellobiose, sucrose and salicin, but not lactose or galactose. The only exception was *Lact. helveticus* N720, which additionally utilized galactose, thereby resembling another metabolically versatile *Lact. helveticus* strain R0052 (Naser *et al.* 2006).

Lactobacillus 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 amino acids from large proteins such as casein (Christensen *et al.* 1999). However, none of the *Lact. helveticus* strains we isolated from sourdough were able to hydrolyse casein.

Assumingly genomic rearrangements have occurred in the adaptation process of *Lact. helveticus* to specific environments. *Lactobacillus helveticus* genome encompasses more than 200 insertion sequence (IS) elements (Callanan *et al.* 2008) that are responsible for the heterogeneity among *Lact. 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 *Lact. helveticus* N92, N720 and E96 genomes is in progress to resolve the adaptation mechanisms to the sourdough environment.

Lactobacillus helveticus remained dominating in the microbial population of industrial sourdough during 7 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 (*Lact. helveticus*, *Lact. panis* and *Lact. pontis*) and also *Lact. vaginalis* were detected with DGGE analysis. Representatives of *Lact. vaginalis* were also detected in the lyophilized starter used for renewing the sourdough cycle.

Culture-independent methods, like DGGE, have several advantages compared with culture-dependent methods in the study of sourdough, because LAB from continuously cycled sourdough can have low colony forming ability (De Vuyst *et al.* 2002; Iacumin *et al.* 2009). Also, media used for the isolation of sourdough lactobacilli quantitatively and qualitatively influence 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 10 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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Supporting Information

Additional Supporting Information may be found in the online version of this article:

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 analysed samples.

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Microbial composition and stability of rye sourdoughs from four industrial bakeries

Ene Viiard [1, 2], Marianna Bessmeltseva [1, 2], Jaak Simm [3], Tiina Talve [1], Anu Aaspõllu [3], Toomas Paalme [1, 2], and Inga Sarand [1, 2, 4]

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

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

[3] Centre for Biology of Integrated Systems, Tallinn University of Technology, Akadeemia tee 15A, Tallinn, Estonia

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

Contact: Inga Sarand, e-mail: inga@tftak.eu; address: Akadeemia tee 15A, 12618, Tallinn, Estonia

Running headline

Microbial composition of industrial rye sourdoughs

Abstract

Aims: To identify the composition of lactic acid bacteria in four industrial rye sourdoughs propagated using different parameters and to evaluate the stability of the microbial communities during backslopping.

Methods and Results: Bacteria within the sourdough communities were identified and their dynamics was monitored using plating, denaturing gradient gel electrophoresis, and metagenomic sequencing of 16S rRNA gene amplicons. Sourdough propagation at elevated temperature ensured dominance of *Lactobacillus amylovorus* and *Lactobacillus frumenti* or *Lactobacillus helveticus*, *Lactobacillus pontis* and *Lactobacillus panis*, while propagation at ambient temperature combined with a short fermentation cycle selected for *Lactobacillus sanfranciscensis*, *Lactobacillus pontis,* and *Lactobacillus zymae* or *Lactobacillus pontis, Lactobacillus helveticus,* and *Lactobacillus zymae.* The ratio of these species displayed a seasonal dependence when employing room-temperature propagation.

Conclusions: Controlled propagation parameters stabilize the microbial communities within sourdough and consequently result in stable chemical properties.

Significance and Impact of Study: This is the first study that evaluates the diversity and stability of LAB within industrial rye sourdoughs in the Baltic region using contemporary identification methods. While most dominant species identified are typical for rye sourdough, *L. helveticus* was identified as a dominant species in the sourdough of two bakeries even though it is not commonly found in cereal environments.

Introduction

Sourdough is a mixture of flour and water that is fermented with lactic acid bacteria (LAB) and yeast. Traditional sourdoughs are propagated by backslopping over many decades (Hansen 2004; De Vuyst and Neysens 2005). During backslopping, a portion of mature sourdough is mixed with fresh flour and water and fermented into new a new batch of sourdough. In mature sourdough, LAB microbiota is prevalent (>8 log CFU g^{-1}) and is mainly dominated by members from the genus *Lactobacillus* (Lonner 1988; Gobbetti *et al.* 1994; Vera *et al.* 2011). Both homo- and heterofermentative species are often found in sourdough

environments while yeast are usually found in lower numbers (<7 log CFU g^{-1}) or are absent (Salovaara and Savolainen 1984; Vera *et al.* 2011). Generally, the ratio of LAB CFUs to yeast CFUs is 100:1 in mature sourdoughs.

Over 50 different species of LAB have been isolated from sourdoughs of different origin (De Vuyst *et al.* 2014), however, most studies on sourdough have focused on the wheat variety with much less attention paid to sourdoughs made from other flours (De Vuyst *et al.* 2014). Despite this large number of species identifications, mature sourdoughs typically contain only two or three dominant species. This includes rye sourdoughs, which are used to make rye bread; a staple of the Nordic diet. The LAB species most often encountered in rye sourdoughs are *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus sanfranciscensis,* and *Lactobacillus acidophilus* (Rosenquist and Hansen 2000; Müller *et al.* 2001; De Vuyst and Neysens 2005; Weckx *et al.* 2010).

Sourdoughs can be classified into three types according to the technology used for their production (Böcker *et al.* 1995; Vogel *et al.* 1999; Hammes and Gänzle 1998; De Vuyst and Neysens 2005; Corsetti *et al.* 2007). Type I sourdoughs are produced using a traditional method based on daily renewal. Type II sourdough processes are used in large-scale production and make use of semi-fluid and easily manageable sourdoughs. Long term continuous propagations are common in type II sourdough processes. Type III sourdoughs are usually initiated by starter cultures and are dried before use. These sourdoughs are commonly used as taste and aroma enhancers.

A sourdough cycle can be started by either the spontaneous fermentation of flour, inoculation with mature sourdough, or using a starter culture (Hansen 2004). The baking industry currently tends to initiate sourdough fermentation with defined commercial starter cultures with specific properties (De Vuyst and Neysens 2005). Unfortunately, those strains may not

adapt to the sourdough propagation scheme in the bakery and are often not competitive enough in the long term to fight off LAB that enter the process from either the flour or the bakery environment. Therefore, to maintain a desirable microbial community, the sourdough cycle is frequently restarted (Meroth *et al.* 2003; Siragusa *et al.* 2009). The microbial composition of sourdoughs is also affected by the process technology and applied conditions (fermentation temperature and time, inoculation rate and dough yield), production environment, and type of flour (De Vuyst and Neysens 2005; Scheirlinck *et al.* 2007; Minervini *et al.* 2012; Minervini *et al.* 2014).

There is limited information regarding the composition and stability of sourdoughs used in large-scale industrial production. The traditions of sourdough preparations and bread making are region-dependent, which is reflected also in sensory characteristics of industrially prepared breads (Valjakka *et al.* 2003). The aims of this work are i) to compare LAB communities in sourdoughs that originate from industrial bakeries of different scales that apply different sourdough propagation parameters but use the same rye flour from the same mill and ii) to evaluate the stability of these microbial communities and their influence on the chemical characteristics of the sourdough over many months of daily renewal using different propagation parameters. Both culture dependent and culture independent methods were used to characterize the microbial communities within the sourdough samples. This is the first study that applies contemporary microbiology methods to assess the microbial diversity and stability of industrial rye sourdoughs in the Baltic region.

Materials and methods

Sourdough samples from industrial bakeries

Sourdoughs from four bakeries were studied and are referred to as A_{bakery}, B_{bakery}, C_{bakery}, and D_{bakery}. Two of the sourdoughs are type II (A_{bakery} and B_{bakery}) and two are type I sourdoughs (C_{bakery} and D_{bakery}). All four bakeries used flour of the same origin (Tartu Mill AS, Estonia), but made use of different sourdough propagation parameters (presented in Table 1). In A_{bakery} and B_{bakery} the sourdough fermentation temperature was precisely controlled while C_{bakery} and D_{bakery} propagated sourdoughs at room temperature which fluctuated seasonally.

A_{bakery} is the oldest and foremost industrial bakery in Estonia and currently holds nearly 40 % market share. The sourdough process in A_{bakery} was initiated with a freeze-dried sourdough made from a well fermented sourdough produced six years previously at the same bakery (sample A0). The renewal cycle for this sourdough involves backslopping and fermentation at 32°C for 10 h (Table 1).

B_{bakery} is a major industrial bakery that holds approximately 20 % of the market share. Their sourdough had been successfully propagated for three years (sample Bs) and was then renewed from fresh cooled sourdough starter sourced from another bakery (sample B0). Their sourdough is routinely propagated at 42–44°C for 16 hours (Table 1).

C_{bakery} was a small bakery, which halted rye bread production in 2010. They propagated a type I sourdough at room temperature with a cycle time of 4 h (Table 1) for small-scale bread production. Their sourdough fermentation was initiated with a freeze-dried commercial starter (C0) one year before the first sourdough sample was collected.

D_{bakery} is also a small-scale bakery that holds 2–3 % market share. Their sourdough is of type I and had been initiated in the 1980's from a commercial starter (probably the same starter as

C0 in C_{bakery}). Prior to the changes introduced during this study, their sourdough was routinely fermented at room temperature for 4–5 h (Table 1). During breaks in production, the sourdough was mixed with flour to lower the water activity and was stored at room temperature for up to 36 h. To improve both the stability of their sourdough and optimize their sourdough production cycle, D_{bakery} adopted new propagation parameters with a well-controlled fermentation temperature. The cycle was extended from 4 h to 12 h and the fermentation temperature was increased to 30°C. In addition, the inoculum size was lowered from approximately one third to 10 %. During breaks in production, the sourdough is now cooled to 4°C and stored for up to 36 h. Sourdough sample D4 was taken immediately before adopting the new sourdough propagation cycle. Sample D5 was collected from sourdough that had been propagated for five months following the new procedure.

Chemical analyses of sourdoughs

To measure the pH and total titratable acidity of each sourdough sample, 5 g of sourdough was homogenized with 45 ml of distilled water. The pH and total titratable acidity (TTA) were measured with Food and Beverage Analyzer D22 (Mettler-Toledo International Inc., USA). Total titratable acidity is given as ml of 0.1 N NaOH used to titrate 10 g of sourdough sample to pH 8.5. All measurements were performed in triplicate.

High performance liquid chromatography (HPLC) was employed to determine the content of sugars (fructose and mannitol, glucose, maltose and sucrose) in the sourdoughs. HPLC Separations Module Waters 2695 Alliance was used with a Refractive Index Detector 2414 (both from Waters Corporation, USA) and column BioRad HPX-87H Organic Acid Analysis Aminex Ion Exclusion Column 300 × 7.8 mm (Bio-Rad Laboratories Inc., USA). A sulfuric acid solution (0.008 N) was used as an eluent at a flow rate of 0.6 ml min^{-1} measured at 35°C.

Enumeration of lactic acid bacteria

The number of culturable LAB in each sourdough sample was determined by plate counting using 5 g of sourdough mixed with 45 ml of sterile 0.85 % NaCl solution. A series of decimal dilutions were plated on MRS agar (LabM, UK). In addition, sample C3 was plated on an mMRS agar (MRS with added 2 % v/v maltose; pH 5.6) and SDB agar (2 % v/v maltose; 0.03 % Tween80; 0.5 % trypticase; pH 5.6) (Kline *et al.* 1971). Plates from A_{bakery}, C_{bakery} and D_{bakery} were incubated at 30°C , while those from B_{bakery} were incubated at 42°C; all under anaerobic conditions. A BD GasPak EZ System (Becton Dickinson Microbiology Systems, USA) was used to maintain an anaerobic environment.

DNA extraction from LAB isolates and sourdough samples

For Rep-PCR analysis, DNA was extracted from isolated colonies on FTA membrane cards (Whatman Inc., USA) using the method provided by the manufacturer. A GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, Inc., USA) was used for DNA extraction from sourdough samples from B_{bakery}, C_{bakery}, and D_{bakery} according to the manufacturer's instructions. A modified phenol-chloroform extraction was used for samples taken from A_{bakery} (Viiard *et al.* 2013).

Fingerprint typing of isolates with Rep-PCR

Rep-PCR with primer $(GTG)_5$ (5'-GTGGTGGTGGTGGTG-3') (Microsynth, Switzerland) was performed as described by Viiard *et al.* (2013) with slight modification; all PCR components were sourced from Solis BioDyne (Estonia). Share of each LAB fingerprint type within selected isolates was calculated as a percentage of the colonies analyzed.

Identification of LAB by 16 rRNA gene sequence analysis

One to two representatives of each fingerprint type group detected using Rep-PCR were selected for 16S rRNA gene analysis. 16S rRNA gene fragments were amplified using the universal primers 27f-YM (Frank *et al.* 2008) and 16R1522 (Weisburg *et al.* 1991) followed by column purification of the amplified fragment with a GeneJET PCR Purification Kit (Thermo Scientific Inc., USA). The fragments obtained were subjected to Sanger sequencing. The partial 16S rRNA gene sequences obtained (approximately 700 bp) were searched against GenBank database using the BLAST algorithm (Basic Local Alignment Search Tool, National Center for Biotechnology Information, USA).

Denaturing Gradient Gel Electrophoresis (DGGE) analysis of microbial communities

To monitor the dynamics of LAB communities within sourdoughs denaturing gradient gel electrophoresis (DGGE) analysis was performed. DNA was amplified using primers F357-GC and 518R as described by (Gafan and Spratt 2005). For yeast, the primers U1GC and U2 were used to carry out the PCR reaction, as described by Meroth *et al.* (2003). Polyacrylamide gel (8 % acrylamide-N,N'-methylenebisacrylamide; 37.5:1) with a gradient from 35 to 70 % urea and formamide (100 % corresponding to 40 % v/v formamide and 7 M urea) was used. Electrophoresis was performed with an INGENY phorU (Ingeny BV International, The Netherlands) at a constant voltage of 70 V at 60°C for 17 h. For yeast, a gradient of 30–50 % was used and electrophoresis was performed at a constant voltage of 130 V at 60°C for 4.5 h. The gels were stained with ethidium bromide and digitized using an ImageQuant 400 system (GE Healthcare, USA). Bands of interest were excised and DNA within these bands was eluted by incubation in a TE buffer overnight at 4°C. The eluted DNA was reamplified using

the same primers without the GC-clamp. The DNA fragments obtained were sequenced using Sanger sequencing.

ITS sequence analysis of yeast community

To amplify internal transcribed spacer (ITS) regions the primers ITS1f (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Microsynth AG, Switzerland) were used (Gardes and Bruns 1993). All PCR reactions were performed in a volume of 25 µl containing 50 ng of DNA, 5 µl of 5 X HOT FIREPol Blend Master Mix (Solis Biodyne, Estonia) and 20 pmol of each primer with the remaining volume consisting of distilled water. Amplified fragments were visualized in a 2 % agarose gel. The resulting DNA bands were cut out and the DNA was extracted using a Gene JET Gel Extraction kit (Thermo Scientific Inc., USA) using the method provided by the manufacturer. The obtained sequences of ITS regions were searched against GenBank database using the BLAST algorithm (Basic Local Alignment Search Tool, National Center for Biotechnology Information, USA).

Pyrosequencing of bar-coded 16S rRNA gene amplicons

Universal primers 8F and 357R were used for PCR amplification of the V2–V3 hypervariable regions of 16S rRNA genes (McKenna *et al.* 2008; Armougom and Raoult 2009). The amplicon mixtures were pyrosequenced using a 454 GS FLX+ System (Roche 454 Life Sciences, USA).

The 454 pyrosequencing data was processed using MOTHUR v.1.32.1 (Schloss *et al.* 2009) according to standard operating procedures (Schloss *et al.* 2011). PCR errors and reads shorter than 150 bp or containing more than eight homopolymers were removed from the dataset. Sequences were aligned to the SILVA reference 16S rRNA database (Prusse *et al.* 2007).

Operational Taxonomic Units (OTUs) were defined using an average neighbor clustering algorithm based on 97 % sequence identity. Normalized OTU counts at 500 reads were calculated with the R (version 3.0.3) software package "vegan" version 2.0-10. In addition, the rate of new OTUs at 500 reads was calculated. The closest match to each OTU within the Greengenes 16S rRNA gene database was identified using BLAST with a minimum of 97 % similarity. To estimate the beta-diversity, non-metric multidimensional scaling (NMDS) was conducted using Yue and Clayton distances (Yue and Clayton 2005) within MOTHUR and results were visualized in R.

Nucleotide sequence accession number

Pyrosequencing data was submitted to the GenBank database under accession numbers KM972414 - KM972548.

Results

Chemical analysis of industrial rye sourdoughs

All pH and TTA measurements are presented in Table 2. B_{bakery} sourdough was characterized by a high acidity (over 30 TTA units) in all samples excepting the initiating starter dough B0. Significant seasonal fluctuations in both pH and TTA occurred in the sourdoughs from C_{bakery} and D_{bakery}. Sample D1 was taken in February, when temperature of the sourdough was 19°C. The concentration of fructose and mannitol, and disaccharides was generally higher in B_{bakery} compared with A_{bakery}, especially after renewal from fresh starter dough (samples B1, B2, B4) (Figure 1). The fructose and mannitol concentrations in the sourdough sample Bs from B_{bakery} was initially low, however, it dramatically increased after renewal of the sourdough cycle with fresh sourdough B0. Sourdoughs propagated with a shorter cycle (4 h) in C_{bakery} and

D_{bakery} contained more glucose at the end of each fermentation cycle compared with both A_{bakery} and B_{bakery}. Also, the concentration of fructose and mannitol (C_{bakery}) and maltose and sucrose (D_{bakery}) were higher in these type I sourdoughs. After increasing the fermentation temperature and time in D_{bakery}, a minor decrease in the concentration of glucose within the sourdough was observed.

Microbial analysis of industrial rye sourdoughs

Sourdough samples from A_{bakery} were monitored for over eight months after renewal from a freeze-dried starter (previously published in Viiard *et al.* 2013). The dominating LAB in the freeze-dried starter dough belonged to species *Lactobacillus helveticus*, *Lactobacillus panis* and *Lactobacillus pontis* (Figure 2). It was shown that during continuous propagation of sourdough in A_{bakery} the proportion of *L. helveticus* colonies decreased, and that of *L. panis* and *L. pontis* increased. DGGE analysis, however, revealed *L. helveticus* as a dominant species during over eight months of propagation (Figure 3). The metagenome analysis performed in the current work confirmed that the microbial community within the A_{bakery} sourdough was remarkably stable after initiation using the freeze-dried starter over eight months of daily renewal (Figure 4). Supplementary information regarding bacterial community data is provided in Table S1.

DGGE analysis of amplified 28S rRNA (Figure 5) and ITS sequence analysis (data not shown) were performed to identify the yeast species present in the sourdough. Both methods showed that *Kazachstania telluris* is the only yeast species within both the freeze-dried starter dough and all other A_{bakery} sourdough samples.

The sourdough from B_{bakery} had been successfully propagated for over three years. After the first sample (Bs) was collected the sourdough was renewed using fresh sourdough sourced from another bakery (sample B0). The stability of B_{bakery} sourdough was monitored for six

months after renewal. The results of culture dependent analysis revealed that both *Lactobacillus amylovorus* and *Lactobacillus frumenti* are dominant species within all sourdough samples collected from B_{bakery} (Figure 2). The same fingerprint type of dominant *L. amylovorus* was detected throughout the study (data not shown). *L. panis* entered the sourdough cycle with the new starter dough and remained within the sourdough at low counts during future propagation cycles. The culture dependent results were confirmed by DGGE analysis (Figure 3) and metagenome analysis (Figure 4). *L. panis* remained undetectable by DGGE analysis while pyrosequencing of the 16S rRNA gene amplicons revealed additional OTUs belonging to *Lactobacillus* sp. that entered the sourdough from starter dough B0 (Figure 4). No yeast species were detected in B_{bakery} sourdough.

In C_{bakery}, the sourdough fermentation was initiated with a commercial freeze-dried starter one year before our study. Bacteria from the species *L. plantarum* and *Lactobacillus casei*/*paracasei* were isolated by plating on MRS media from the starter sample (C0) (Figure 2). However, in the sourdough sample collected one year after the renewal (sample C1) only bacteria from the species *Lactobacillus zymae*, *L. fermentum*, *L. pontis*, *Leuconostoc lactis* and *Pediococcus acidilactici* were detected (Figure 2). In the sourdough samples taken 21 and 28 months after renewal (samples C2 and C3), only *L. zymae* and *L. pontis* species were identified. In contrast, DGGE analysis revealed *L. pontis* as the only species in the commercial freeze-dried starter and *L. pontis* and *L. sanfranciscensis* in all sourdough samples (Figure 3). These results were largely confirmed by metagenome analysis of C_{bakery} samples (Figure 4) excepting the large proportion of *L. zymae* found in C1 sample.

To confirm the presence of *L. sanfranciscensis* in the sourdough using a culture dependent method, two additional media, SDB and mMRS, were tested using the C3 sample. mMRS appeared to be the only media where *L. sanfranciscensis* could be isolated after an extended incubation time (72 h). While the ratio of LAB species on MRS medium was 80 % *L. pontis*

and 20 % *L. zymae,* the corresponding ratios using mMRS medium were 72 % *L. sanfranciscensis*, 17 % *L. pontis* and 11 % *L. zymae*.

Candida humilis was the only yeast species detected in C_{bakery} sourdough using both DGGE analysis of an amplified region of 28S yeast rRNA (Figure 5) and ITS sequencing (data not shown). No yeast species were detected in the freeze-dried starter used to initiate the sourdough process in C_{bakery}.

The sourdough samples from D_{bakery} contained LAB species within *L. zymae*, *L. pontis* and *L. helveticus* (Figures 2, 3 and 4). The relative proportion of these species in a given sourdough sample was found to depend on the ambient temperature in the bakery. In the wintertime (sample D1), growth of *L. zymae* was favored, while *L. pontis* and *L. helveticus* dominated in the samples taken during warmer periods (samples D2, D3 and D4). The latter two species also dominated in the sourdough (sample D5) after adopting the new propagation parameters with a controlled elevated fermentation temperature (Supplementary Figure S1). Both DGGE and ITS sequence analysis revealed that the yeast species *C. humilis* dominates within D_{bakery} sourdough (Figure 5).

Beta-diversity analysis

Two-dimensional non-metric multidimensional scaling (NMDS) provided a stress value of 0.156 and an R2 value of 0.885 (Figure 6). The stress value decreased to 0.064 and the R2 value increased to 0.977 when calculating the NMDS with three dimensions (data not shown). NMDS of the pyrosequencing data places sourdough samples from A_{bakery} and B_{bakery} in two closely grouped clusters, which illustrates the stability of both sourdough propagation processes. The same analysis shows that samples from both C_{bakery} and D_{bakery} change over time and thus indicate that the communities within these sourdoughs are less stable (Figure 6). The freeze-dried starter C0 that contained *L. pontis* groups together with D_{bakery} samples

where *L. pontis* is prevalent. A_{bakery} and D_{bakery} sourdough samples can be found in the same quadrant of the NMDS plot because they both contain *L. helveticus* and *L. pontis*. B_{bakery}, which employs a higher fermentation temperature, differs significantly from all other sourdoughs.

Discussion

This study evaluates the stability of both the microbial community and chemical properties of rye sourdoughs from four Estonian bakeries. The bakeries use flour from the same source, but employ different propagation parameters varying at different scales of production. Our data show that controlling the propagation parameters stabilizes the count and distribution of dominant LAB species in rye sourdoughs during long term backslopping. The number of culturable LAB fluctuated in sourdoughs fermented at ambient temperature. High LAB numbers correlate with high titratable acidity and low pH, which are both characteristic to mature rye sourdough and prerequisite for producing rye bread with desirable sensory properties. Because lactic acid production is directly linked to the fermentation temperature, B_{bakery} sourdough samples possessed the highest acidity compared with sourdoughs from the other three bakeries. In contrast, low ambient fermentation temperatures during the winter months, combined with a short fermentation cycle resulted in low LAB counts and insufficient acid production in both C_{bakery} and D_{bakery}. Adoption of a higher fermentation temperature of 30°C by D_{bakery} resulted in the stabilization of LAB numbers and pH even in this small scale bakery using traditional sourdough.

The same tendencies were also observed for the stability of LAB communities. The LAB communities remained stable in sourdoughs fermented at controlled conditions, whereas the fractional composition of LAB in sourdoughs fermented at ambient temperature was seasonally dependent. Sourdoughs in both large-scale bakeries studied (A_{bakery} and B_{bakery})

were initiated from LAB communities that had previously been adapted to the propagation parameters employed in these bakeries. A_{bakery} initialized their process with a freeze-dried sourdough they had prepared six years earlier, while B_{bakery} used fresh sourdough from another bakery. *L. helveticus* or *L. amylovorus* together with *L. frumenti* formed the dominant communities in these two bakeries, respectively. *L. amylovorus* and *L. frumenti* have previously been identified as prevalent species in other industrial rye sourdoughs propagated at elevated fermentation temperatures (Müller *et al.* 2001; De Vuyst *et al.* 2014). The persistence of *L. amylovorus* in sourdoughs may be attributed to its high amylolytic activity and its ability to produce bacteriocine amylovorin, which is a common feature for representatives of this species (De Vuyst *et al.* 2004; Leroy 2007). The same fingerprint type of *L. amylovorus* was detected in samples collected from B_{bakery} both before and after restarting the sourdough propagation cycle with fresh sourdough sourced from another bakery. This indicates that this *L. amylovorus* strain may have adapted well during continuous propagation.

Although *L. helveticus* is not a common dominant species in sourdoughs (Scheirlinck *et al.* 2007; Viiard *et al.* 2013; De Vuyst *et al.* 2014) we found that it dominated also in the sourdough of a small scale bakery. However, in contrast with A_{bakery}, the sourdough fermentation at ambient temperature in D_{bakery} prevented the stable prevalence of thermophilic *L. helveticus*. Depending on the season, *L. pontis* and *L. zymae* dominated this species. *L. zymae* has previously been found in both Greek and Belgian wheat sourdoughs, which indicates that it is widely spread (De Vuyst *et al.* 2002; Vancanneyt *et al.* 2005). New technological parameters adopted in D_{bakery} (higher fermentation temperature, decreased inoculation rate, prolonged fermentation time, and use of 4°C refrigeration during breaks in production) stabilized the LAB community and caused an increase in the proportion of *L. helveticus* and *L. pontis*. Interestingly, no new species were detected even after five months of

propagation with the new propagation parameters. No notable effects on the utilization of sugars and production of acids were observed, even though prolonged sourdough fermentation time typically selects bacteria that are able to grow under acidic conditions (Vrancken *et al.* 2011). It has been shown that *L. helveticus* strains isolated from A_{bakery} sourdough are characterized by high specific growth rates at low pH, thus indicating acid tolerance (Mihhalevski *et al.* 2011).

Representatives of *L. zymae* and *L. pontis* species were also detected among the dominant population of LAB in C_{bakery}, which utilized sourdough propagation parameters that are very similar to those originally used in D_{bakery}. In contrast with the latter sourdough, *L. sanfranciscensis* was also identified among prevailing bacteria in sourdough samples from C_{bakery}. *L. sanfranciscensis* is frequently found in type I sourdoughs due to its adaptation to sourdough conditions, its small genome, and metabolism (De Vuyst *et al.* 2014). Stable non-competitive association of this maltose-positive LAB species with maltose-negative yeast *C. humilis* exists in traditional sourdoughs (Gänzle *et al.* 1998). *L. sanfranciscensis* species are capable of hydrolyzing maltose by intracellular maltose phosphorylase activity and thereby accumulate glucose in the environment for *C. humilis* to utilize (Stolz *et al.* 1996). *C. humilis* was the only yeast species identified in the sourdoughs of both small-scale productions C_{bakery} and $D_{bakery,}$ although the microbial community of the latter did not contain *L. sanfranciscensis*. The presence of *L. sanfranciscensis* in the sourdough samples from C_{bakery} correlate with high concentrations of fructose and mannitol (De Vuyst *et al.* 2002). *C. humilis* is able to produce fructose from some flour oligosaccharides, which may provide an advantage to LAB that are capable of utilizing fructose as an electron acceptor (Gobbetti *et al.* 1995; Stolz *et al.* 1995), and lessens the competition for substrates between LAB and yeasts (Gobbetti and Corsetti 1996).

Co-existence of *L. helveticus* with the yeast species, *K. telluris*, was detected in the sourdough from A_{bakery}. *K. telluris* (formerly *Saccharomyces telluris*, *Arxiozyma telluris*) is mainly known from infections in rodents, however, it has also been isolated from soil (Kurtzman *et al.* 2005). Occurrence of this species in sourdough has not been previously reported, however, this thermophilic yeast species is able to ferment glucose and grow on glucose, ethanol, and lactic acid (Kurtzman *et al.* 2005). Because our identification is based only on culture-independent methods (sequencing of 28S rRNA and ITS regions) further research should be carried out to evaluate the role of this yeast species in this sourdough community. The high fermentation temperature in B_{bakery} prevented the development of yeast in the sourdough.

The culture independent methods applied in this study enabled us to identify LAB species from sourdough samples that were difficult to cultivate on the selected medium, especially representatives from *L. helveticus* and *L. sanfranciscensis.* It has been previously shown that many sourdough LAB are sensitive to oxygen and/or have complex nutrient requirements (Vera *et al.* 2009; Mihhalevski *et al.* 2011). A wide variety of media should therefore be used to isolate sourdough LAB because there is no universal medium that is suitable for all LAB. Culture independent methods such as DGGE and metagenomic sequencing enable one to detect LAB that are difficult to culture on common laboratory media. High throughput sequencing also allows for species identification at the sub-population level and provides quantitative information regarding the fractional makeup of species within sourdough. Based on these methods new information concerning composition of industrial rye sourdoughs in Baltic region was obtained in this study.

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396 Conflict of interest

397 No conflict of interest declared.

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Table 1. Sourdough propagation parameters applied in four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}).

Bakery	**A_{bakery}**	**B_{bakery}**	**C_{bakery}**	**D_{bakery}**
Fermentation temperature	32°C	42–44°C	RT (up to 28°C)	RT (19–30°C)
Inoculation rate	10 %	6 %	~ 33 %	~ 33 %
Fermentation time	10 h	16 h	~ 4 h	~ 4 h
Dough yield	250	400	~ 200	~ 200
Starter used	Freeze-dried sourdough culture	Active sourdough starter	Commercial freeze-dried starter	Commercial freeze-dried starter
Analyzed samples	A0 - freeze-dried sourdough A1 - 1.2 months A2 - 3.5 months A3 - 4.5 months A4 - 8.5 months	Bs - 3 years propagated sourdough B0 - fresh sourdough starter B1 - 0.25 months B2 - 1 months B3 - 2.5 months B4 - 6 months	C0 - freeze-dried commercial starter C1 - 12 months C2 - 21 months C3 - 28 months	D1 - approx. 30 years propagated sourdough D2 – 3 months D3 – 5 months D4 – 16 months (before transfer to 30°C) D5 – 21 months (5 months after transfer to 30°C)

Table 2. Chemical properties and microbiological characteristics of rye sourdoughs from four industrial bakeries in Estonia (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}): pH, total titratable acidity (TTA) and total number of culturable lactic acid bacteria obtained from anaerobically incubated MRS plates.

Sample	pH	TTA ml 0.1 N NaOH / 10 g	log CFU g^{-1}
A0	NA*	NA	NA
A1	3.67 ± 0.06	22.30 ± 0.56	8.82 ± 0.07
A2	3.71 ± 0.01	18.89 ± 0.05	9.04 ± 0.06
A3	3.63 ± 0.04	21.30 ± 0.41	8.84 ± 0.07
A4	3.53 ± 0.02	21.84 ± 0.30	9.08 ± 0.03
Bs	3.60 ± 0.05	31.33 ± 0.13	8.63 ± 0.16
B0	3.40 ± 0.12	21.60 ± 0.58	8.56 ± 0.14
B1	3.63 ± 0.08	30.23 ± 0.02	8.11 ± 0.06
B2	3.57 ± 0.06	31.38 ± 1.27	8.94 ± 0.08
B3	3.57 ± 0.05	34.51 ± 1.28	8.93 ± 0.24
B4	3.58 ± 0.06	33.18 ± 1.09	8.85 ± 0.05
C0	NA	NA	NA
C1	4.11 ± 0.09	16.50 ± 1.11	6.56 ± 0.07
C2	4.00 ± 0.07	18.20 ± 0.03	6.64 ± 0.01
C3	4.18 ± 0.11	17.10 ± 0.58	8.28 ± 0.03
D1	4.28 ± 0.06	16.94 ± 1.08	8.00 ± 0.03
D2	3.96 ± 0.16	18.30 ± 1.06	8.80 ± 0.08
D3	3.86 ± 0.06	23.19 ± 1.10	9.05 ± 0.12
D4	4.12 ± 0.11	17.85 ± 0.03	8.01 ± 0.11
D5	3.78 ± 0.07	18.98 ± 0.03	8.31 ± 0.08

* NA – not acquired

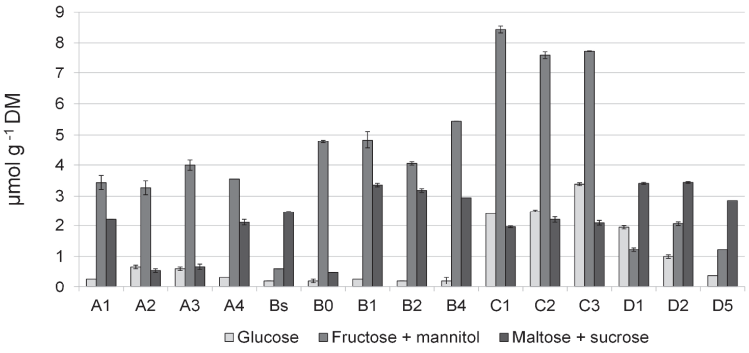

Figure 1. Chemical analysis of industrial rye sourdough samples of four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}). Concentrations of glucose, fructose and mannitol, maltose and sucrose, are given in µmol g^{-1} dry matter (DM).
281x126mm (300 x 300 DPI)

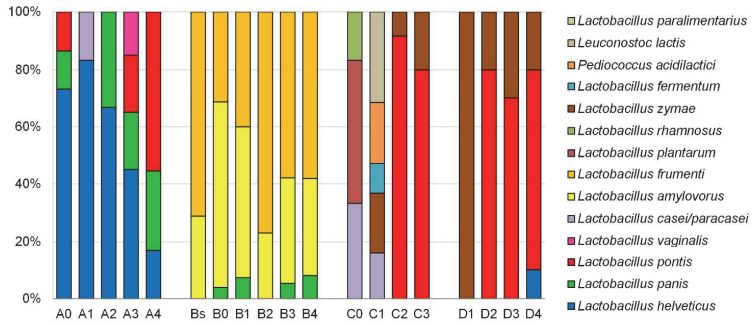

Figure 2. Microbial composition of industrial rye sourdoughs of four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) as determined by Rep-PCR analysis of colonies isolated from MRS plates. The share of each LAB fingerprint type within selected isolates is calculated as a percentage of the colonies analyzed.
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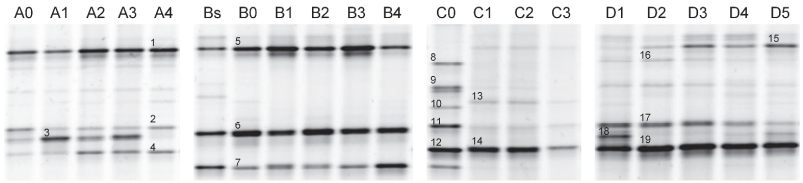

Figure 3. Stability of bacterial communities in industrial rye sourdoughs of four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) as determined by DGGE analysis of the 16S rRNA gene amplicons. 1 – *Lactobacillus helveticus*; 2 – *Lactobacillus panis*; 3 – Cereal chloroplast DNA; 4 – *Lactobacillus pontis*; 5 – *Lactobacillus amylovorus*; 6 – Cereal chloroplast DNA; 7 – *Lactobacillus frumenti*; 8 –10 – *Lactobacillus pontis*; 11 – Cereal chloroplast DNA; 12 – *Lactobacillus pontis*; 13 – *Lactobacillus sanfranciscensis*; 14 – *Lactobacillus pontis*; 15 – *Lactobacillus helveticus*; 16 – *Lactobacillus pontis*; 17 – Cereal chloroplast DNA; 18 – *Lactobacillus zymae*; 19 – *Lactobacillus pontis*. Identification was carried out using BLAST by finding the closest match to sequences within the GenBank database at a minimum similarity of 97 %. Sample codes are presented in Table 1.
434x100mm (300 x 300 DPI)

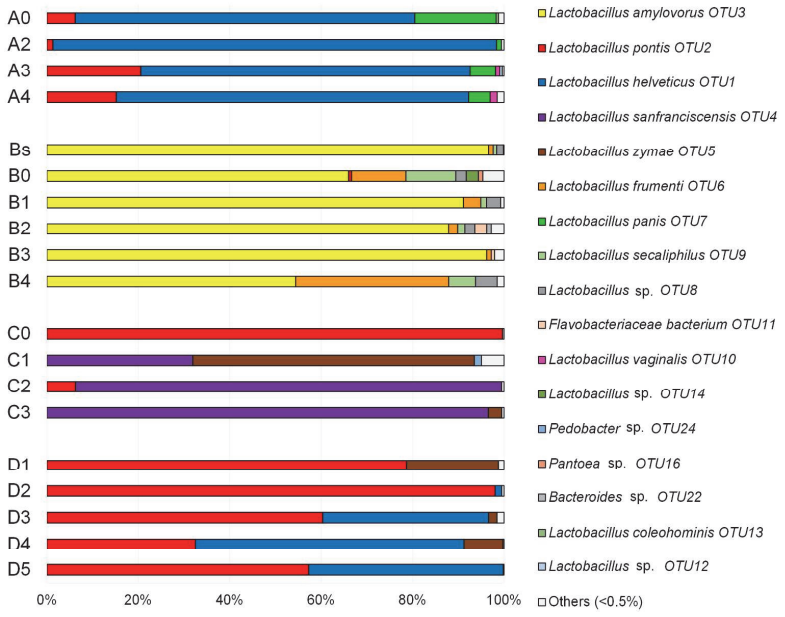

Figure 4. Species identification and distribution within rye sourdough samples from four industrial bakeries in Estonia (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) using pyrosequencing analysis. OTUs that form less than 0.5 % are grouped as 'Others'. OTU sequences were submitted to GenBank database under accession numbers KM972414 - KM972548.
211x169mm (300 x 300 DPI)

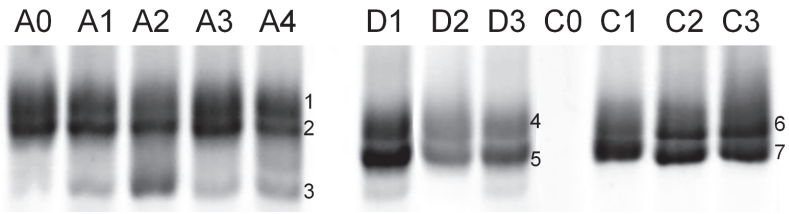

Figure 5. Identification of yeast communities within industrial rye sourdoughs from three Estonian bakeries (A_{bakery}, C_{bakery} and D_{bakery}) using DGGE analysis of 28S rRNA gene amplicons. 1, 2 – *Kazachstania telluris*; 3 – Cereal DNA; 4, 5, 6, 7 – *Candida humilis*.
217x58mm (300 x 300 DPI)

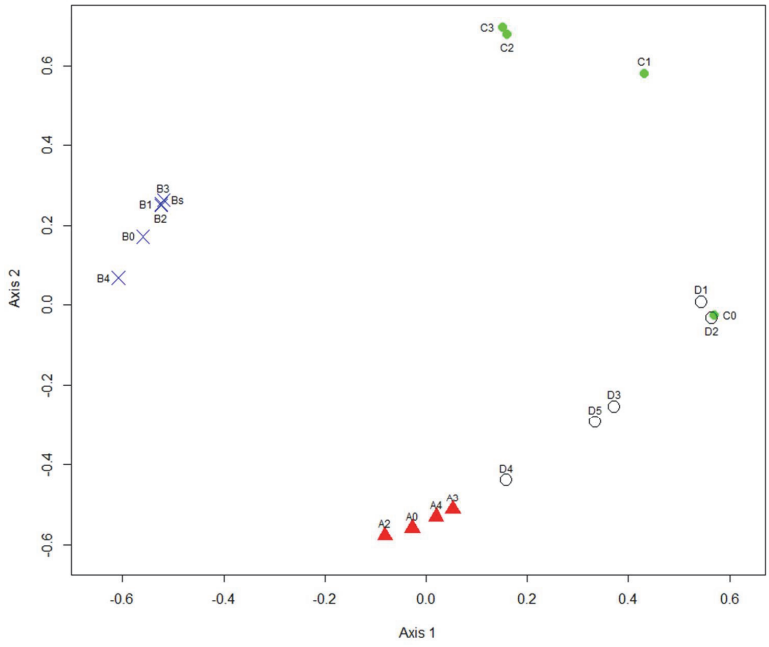

Figure 6. Two dimensional non-metric multidimensional scaling (NMDS) of sourdough samples from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) with a stress value of 0.156 and an R2 value of 0.885. 78x70mm (300 x 300 DPI)

Publication III

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Evolution of Bacterial Consortia in Spontaneously Started Rye Sourdoughs during Two Months of Daily Propagation

Marianna Bessmeltseva[1,2☯], Ene Viiard[1,2*☯], Jaak Simm[3], Toomas Paalme[1,2], Inga Sarand[1,2,4]

1 Competence Center of Food and Fermentation Technologies, Tallinn, Estonia, **2** Department of Food Processing, Tallinn University of Technology, Tallinn, Estonia, **3** Centre for Biology of Integrated Systems, Tallinn University of Technology, Tallinn, Estonia, **4** Department of Gene Technology, Tallinn University of Technology, Tallinn, Estonia

Abstract

The evolution of bacterial consortia was studied in six semi-solid rye sourdoughs during long-term backslopping at different temperatures. Each rye sourdough was started spontaneously in a laboratory (dough yield 200), propagated at either 20°C or 30°C, and renewed daily at an inoculation rate of 1:10 for 56 days. The changes in bacterial diversity over time were followed by both DGGE coupled with partial 16S rRNA gene sequencing and pyrosequencing of bar-coded 16S rRNA gene amplicons. Four species from the genus *Lactobacillus (brevis, crustorum, plantarum,* and *paralimentarius)* were detected in different combinations in all sourdoughs after 56 propagation cycles. Facultative heterofermentative lactic acid bacteria dominated in sourdoughs fermented at 30°C, while both obligate and facultative heterofermentative LAB were found to dominate in sourdoughs fermented at 20°C. After 56 propagation cycles, *Kazachstania unispora* (formerly *Saccharomyces unisporus*) was identified as the only yeast species that dominated in sourdoughs fermented at 20°C, while different combinations of strains from four yeast species (*Kazachstania unispora*, *Saccharomyces cerevisiae*, *Candida krusei* and *Candida glabrata)* were detected in sourdoughs propagated at 30°C. The evolution of bacterial communities in sourdoughs fermented at the same temperature did not follow the same time course and changes in the composition of dominant and subdominant bacterial communities occurred even after six weeks of backslopping.

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* E-mail: ene@tftak.eu

☯ These authors contributed equally to this work.

Introduction

Sourdough is a mixture of flour and water fermented with a microbial community mainly consisting of lactic acid bacteria (LAB) and yeasts. LAB dominate the microbial community and are responsible for acid production while yeasts work to leaven the dough [1,2]. Lactic acid fermentation plays an important role in the production of rye bread by both decreasing the activity of α-amylase and improving dough texture [3].

Lactobacillus brevis, Lactobacillus plantarum, and Lactobacillus sanfranciscensis are the LAB species most frequently isolated from rye sourdoughs [2,4–5]. However, Lactobacillus amylovorus, Lactobacillus fermentum, Lactobacillus helveticus, Lactobacillus panis, Lactobacillus pontis, and Lactobacillus reuteri have also been identified as dominant species in rye sourdoughs [4,6–9]. Since rye flours have a generally higher extraction rate than wheat flours [5] rye sourdoughs are characterized by higher buffering capacity compared with wheat and spelt sourdoughs but also higher concentration of mannitol and amino acids [8,10]. Despite this, the same LAB species are able to dominate the microbial communities within mature sourdoughs made with different types of flour under otherwise identical conditions [8,10–11]. Recently, it has been shown that initial differences in the microbial communities found in spontaneously started rye and wheat sourdoughs decreases during backslopping propagations and that a common core microbiota is established [11].

The establishment and stability of microbial consortia in sourdoughs depends on the microbial communities within the raw materials, the chemical composition of the raw materials, and interactions between the microorganisms, together with fermentation parameters such as temperature, inoculum size, dough yield, and fermentation length [12–16]. Previous studies on the dynamics of microbial communities in spontaneously started sourdoughs have stated that the establishment of a stable consortium occurs through a three-stage evolution process within a few days during which one may observe the prevalence of sourdough-atypical LAB, sourdough-typical LAB, and highly adapted sourdough-typical LAB [2,8,10–11]. Most of these studies have been carried out with liquid sourdoughs fermented using continuous stirring and propagated over a short period of time

(maximum two weeks). Traditional Estonian rye sourdoughs can be classified as semi-solid because they have a relatively dense consistency. They are fermented at ambient temperature, which may vary between 18–27°C depending on the season. Because the water content (dough yield) and fermentation temperature are the main factors that affect the composition of the bacterial community [2], we evaluated the establishment of microbial consortia in spontaneously fermented rye sourdoughs with a low dough yield at two different temperatures (20 and 30°C) back-slopped daily for 56 days. The aims of our study are (i) to gain insight into the stability of bacteria communities after their initial establishment in mature sourdough, (ii) to determine the effect of fermentation temperature on the development of bacterial communities in sourdoughs, (iii) to assess the reproducibility of the development of microbial communities in sourdough when applying fermentation conditions with low dough yield.

Materials and Methods

Sourdough Fermentation and Sampling

Sourdough fermentation was initiated in six 400 ml sterile Stomacher circulator bags (Seward Limited, England) by mixing 150 g of rye flour and 150 g of sterile 0.5% NaCl solution. Rye flour (Type 1370, Tartu Mill, Estonia) from a single 50 kg bag was used during the entire experiment. Each sourdough was mixed for 15 minutes at 100 rpm using a Stomacher 400 circulator (Seward Limited, England), the bags were sealed with tape, and positioned vertically into an incubator. Three sourdough batches were fermented at 20°C and another three at 30°C. After 24 hours of fermentation, each sourdough was thoroughly mixed in the circulator for 5 min at 100 rpm and renewed at an inoculation rate of 1:10 by mixing 30 g of sourdough, 135 g of sterile 0.5% NaCl solution, and 135 g of rye flour. In total, each sourdough experienced 56 backslopping cycles. The three sourdoughs fermented at 20°C are referred to as 20-I, 20-II, and 20-III while those fermented at 30°C are referred to as 30-I, 30-II, and 30-III. Day 0 indicates the start of the experiment.

Both the acidity, in units of pH, and total titratable acidity (TTA) were measured using a DL22 Food and Beverage Analyzer (Mettler-Toledo LLC., USA) at the end of each fermentation cycle prior to renewal. Following this, five grams of sourdough was homogenized with 50 ml of distilled water using a Polytron PT2100 homogenizer (Kinematica AG, Switzerland).

Both bacterial plate counts and DGGE analysis were carried out on days 0, 1, 3, 5, 7, 10, 15, 21, 28, 35, 42, and 56 of the experiment. Pyrosequencing was performed on samples from days 1, 3, 5, 7, 21, and 56. The sample from day 0 was taken from the rye flour and water mixture immediately after mixing.

Isolation and Characterization of Lactic Acid Bacteria and Yeasts

Five grams of sourdough were supplemented with sterile 0.85% NaCl solution up to a volume of 50 ml. The mixture was then homogenized by vortexing. Decimal dilutions were plated on to both sourdough bacteria (SDB) agar (maltose, 2.0%; yeast extract, 1%; Tween 80, 0.03%, trypticase 0.6%; pH 5.6) and de Man, Rogosa and Sharpe (MRS) agar (Lab M Ltd, UK) with 100 μg/ml cycloheximide (Sigma-Aldrich, USA).

The plates were incubated at the same temperature the sourdough was fermented at (20 or 30°C). Incubation was carried out for 48 h under anaerobic conditions (AnaeroGen, Oxoid). Colony forming units (CFU) were counted from the agar media using suitable dilutions.

For each of the six sourdough samples collected on day 56, 20 colonies were picked from the MRS and SDB agar plates (ten from each medium) for further analysis by rep-PCR. Colony picking was performed in succession from one sector of the plate. On day 56 samples were also plated on Yeast Extract Peptone Dextrose (YPD) agar (dextrose, 2.0%; peptone 2%; yeast extract, 1%) with 100 μg/ml chloramphenicol (Sigma-Aldrich, USA) and incubated at the same temperature the sample was fermented at (20 or 30°C). Ten colonies per sample were picked in succession from YPD agar plates and analyzed using RAPD-PCR.

Extraction of Whole Genomic DNA

Total DNA extraction was performed using 5 g of sourdough, which had been homogenized by vortexing with 45 ml of sterile physiological solution [10]. This suspension was then centrifuged at 4°C for 5 minutes at 1000×g. The supernatant was collected and centrifuged at 4°C for 15 minutes at 5000×g. Each extraction of whole DNA was performed using a GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich., USA), according to the manufacturer's protocol. Total DNA was also extracted from the rye flour and water mixture immediately after mixing on day 0.

Denaturing Gradient Gel Electrophoresis Analysis

The V3 region of the 16S rRNA gene was amplified from the whole genomic DNA using universal primers F357-GC and 518R as described in [10]. DGGE was carried out using the INGENYphorU system (Ingeny International Bv., Netherlands) as described in Viiard et al. [9]. All clearly visible bands were cut from the gel and incubated in TE buffer (10 mM Tris pH 7.5 and 1 mM EDTA pH 8.0) at 37°C for 1 h. Eluted DNA was reamplified using primers F357 and 518R and sequenced in a commercial facility (Estonian Biocentre, Tartu, Estonia). Nucleotide sequences were analyzed using the BLASTn algorithm together with the GenBank database (National Center for Biotechnology Information, USA).

Pyrosequencing of Bar-coded 16S rRNA Gene Amplicons

Universal primers 8F and 357R were used for PCR amplification of the V1–V2 hypervariable regions of 16S rRNA genes [17]. The amplicon mixtures were pyrosequenced using a 454 GS FLX+ System (Roche 454 Life Sciences, USA) in a university facility (Centre for Biology of Integrated Systems, Estonia).

The resulting pyrosequencing data was analyzed using the software package MOTHUR, version 1.27.0 [18]. Reads shorter than 150 bps were removed from the dataset and the PyroNoise algorithm was used to discard both homopolymer-derived and PCR errors. The remaining sequences were aligned to the SILVA reference 16S ribosomal RNA database [19]. Chimeric sequences were filtered using the UChime method by applying the 'chimera.uchime' procedure in MOTHUR in *de novo* mode, which checks chimeras in each group separately. Operational Taxonomic Units (OTUs) were defined using the average neighbour clustering algorithm within MOTHUR with a 97% similarity threshold. Rarefaction curves and normalized OTU counts at 500 sequences were calculated using the R software package "vegan" version 2.0-7. In addition, we calculated the rate of forming new OTUs when one sequence is added to the set of 500 sequences. The closest match on the species level was found for each OTU using the BLASTn algorithm together with the GenBank database (National Center for Biotechnology Information, USA) with the parameters of 97% similarity and 90% coverage. The relative abundance of OTUs was calculated as the number of sequences for each OTU divided by the total number of bacterial sequences obtained for each sourdough sample.

Statistical Analysis

Plate count data was subjected to Z-tests to both compare the results obtained at 20 and 30°C and to compare results obtained using different media. We tested the hypotheses that the difference between the results obtained at different conditions come from a distribution with mean zero. The nuisance parameter of the z-test is the sum of the standard deviation of the differences between the values obtained at two conditions and the standard deviations of the values obtained at each condition.

Acidity and TTA measurements obtained at 20 and 30°C were compared using a simple two sample Student's t-test for samples with equal variance. The values tested were considered to come from distributions with different mean values when the p-value was below 0.05.

DNA Isolation from Colonies, Rep-PCR Fingerprinting and Partial Sequencing of rRna Genes

Bacterial DNA was extracted from isolated colonies using Whatman indicating FTA MiniCards (GE Healthcare Ltd., UK) using a method provided by the manufacturer. Rep-PCR with primer $(GTG)_5$ followed by agarose gel electrophoresis was performed as described by Viiard et al. [9]. Extraction of yeast DNA was carried out using a PureLink Genomic DNA mini Kit (Invitrogen, USA) using a method provided by the manufacturer. RAPD-PCR with an M13 primer was performed according to Andrighetto et al. [20]. Each fingerprint type was calculated as the ratio of similar fingerprints to the number of colonies analyzed. One or two representatives from each PCR fingerprint group were subjected to Sanger sequencing. The resulting 16S rRNA gene fragments were amplified using universal primers 27f-YM [21] and 16R1522 [22]. Yeasts were identified using standard protocols by amplifying the D1/D2 variable domains of the 26S rRNA gene with primer pair NL1 and NL4 [23]. Amplified fragments were purified with a GeneJET PCR Purification kit (Fermentas, Vilnius, Lithuania). Sequencing of the fragments was conducted at a commercial facility (Estonian Biocenter, Estonia). The resulting gene sequences were compared with the GenBank database using the BLASTn algorithm (National Center for Biotechnology Information, USA).

Evaluation of Carbohydrate Fermentation Profiles

Carbohydrate fermentation profiles of selected LAB and yeast strains were determined with the identification kits API 50 CH and API 20 C AUX using methods provided by the manufacturer (bioMérieux, France).

Results

pH, Total Titratable Acidity, and Bacterial Plate Counts

Spontaneous rye sourdoughs were started at 20°C and 30°C in three parallels. After 24 hours of fermentation at 20°C the average viable count of bacteria increased from 2.0×10^5 CFU/g in the raw flour to 5.7×10^8 while the first fermentation cycle at 30°C resulted in 7.2×10^8 CFU/g (Table 1). After the third backslopping cycle (day 3) the viable count of bacteria in all sourdoughs exceeded 10^9 CFU/g. No significant ($p<0.05$) difference was found in the plate counts obtained from SDB or MRS media.

Fermentation both increased the concentration of acids and lowered pH of the rye sourdoughs. For the fermentation series conducted at 30°C, the maximum TTA value reached (22.5 ± 0.7) occurred on day 10 while the 20°C fermentation series displayed its maximum TTA value (19.5 ± 1.0) on days 11 and 12 (Figure 1). The maximum value for the 30°C series was larger, however, the difference in TTA between sourdoughs fermented at different temperatures diminished during later propagation cycles (Figure 1).

During the first ten cycles, the pH in the sourdoughs fermented at 30°C decreased significantly more compared with those fermented at 20°C (Figure 1). However, during the later stages of backslopping, the acidity did not significantly differ between sourdoughs.

Dynamics of the Bacterial Communities Determined by DGGE of 16S rRNA Gene PCR Amplicons

The highest diversity of species detected using DGGE analysis occurred after the first 24 hours of fermentation (Figure 2A). Over 15 bands were visible, however, only few of the sequences obtained by cutting these bands were identified at a similarity level ≥97% (Table 2). Of those identified, three belong to *Pantoea agglomerans,* one to a *Gamma Proteobacteria* species and one to a *Weissella* species. All of the bands identified were observed at both fermentation temperatures. After the third backslopping, the DGGE patterns of all six sourdoughs were similar to each other and consisted of fragments specific to the LAB genera *Weissella*, *Lactobacillus*, and *Pediococcus*. On day 5 the DGGE patterns of sourdoughs fermented at 20°C had not drastically changed. However, from sourdoughs fermented at 30°C, the bands specific to *Weissella* sp. and *Lactobacillus* sp. had disappeared and another band, identified as *Lactobacillus plantarum,* emerged (Figure 2A).

On day 7, a band specific to *L. plantarum* was also detected in all sourdoughs fermented at 20°C together with another band identified as *Pediococcus pentosaceus*. In addition, a third new band identified as *Lactobacillus curvatus/graminis/sakei* was found in batchs 20-I and 20-II (Figure 2B), however, the DNA sequence obtained from this band did not provide sufficient information to discriminate between these three *Lactobacillus* species even at 99% identity (Table 2). Differences were also found among sourdough batches fermented at 30°C. Fragments specific to *Lactobacillus brevis* and *P. pentosaceus* were seen only in sourdoughs 30-I and 30-III in addition to *L. plantarum*, which was found in all three batches. The DGGE pattern of 30-II had two additional bands, one of which was identified as *L. sakei* (Figure 2B and Table 2).

The diversity of the six bacterial communities continued to decrease during the second and third week of propagation (Figure 2B). On day 21, two major bands specific to the species *L. plantarum* and *L. brevis* were observed in sourdoughs 20-I and 20-III while *L. plantarum* and *Lactobacillus crustorum* were detected in sourdough 20-II. In sourdoughs fermented at 30°C, only one single strong band corresponding to either *Lactobacillus paralimentarius* (30-I) or *L. plantarum* (30-II and 30-III) was observed after cycle 21 (Figure 2B).

The bacterial composition of the sourdoughs was both stable and comparable between batches from day 21 to 35. However, further succession of species occurred after the fifth week of propagation (Figure 2C). On the final day of sampling, sourdoughs fermented at 20°C were comprised of either *L. brevis* and *L. paralimentarius* (sourdough 20-I), *L. brevis*, *L. plantarum* and *L. crustorum* (sourdough 20-II), or *L. brevis* and *L. plantarum* (sourdough 20-III). In sourdoughs fermented at 30°C *L. paralimentarius* was detected in all three sourdoughs either in combination with *L. plantarum* and *L. crustorum* (sourdough 30-I), together with *L. plantarum* (sourdough 30-II) or as a single dominant species (sourdough 30-III).

Table 1. Enumeration of lactic acid bacteria in spontaneous rye sourdoughs during two months of backslopping; viable counts are given as log CFU/g obtained on SDB and MRS media.

	Sourdoughs fermented at 20°C								Sourdoughs fermented at 30°C							
	SDB				MRS				SDB				MRS			
Day	20-I	20-II	20-III	Average at 20°C	20-I	20-II	20-III	Average at 20°C	30-I	30-II	30-III	Average at 30°C	30-I	30-II	30-III	Average at 30°C
0	5.30	5.29	5.32	5.30±0.02	5.31	5.28	5.28	5.29±0.02	5.32	5.30	5.28	5.30±0.02	5.30	5.31	5.30	5.31±0.01
1	8.82	8.70	8.77	8.76±0.06	8.81	8.69	8.76	8.75±0.06	8.82	8.91	8.88	8.87±0.05	8.81	8.91	8.79	8.84±0.06
3	9.35	9.38	9.34	9.36±0.02*	9.28	9.24	9.26	9.26±0.02*	9.10	9.11	9.17	9.13±0.04*	9.05	9.13	9.07	9.08±0.04*
5	9.44	9.49	9.22	9.38±0.14	9.36	9.40	9.28	9.35±0.07*	9.30	9.39	9.34	9.34±0.05	9.17	9.16	9.07	9.13±0.05*
7	9.17	9.02	8.92	9.04±0.13	9.11	8.92	8.88	8.97±0.13	9.12	8.70	8.75	8.86±0.23	9.07	8.96	8.93	8.99±0.08
10	9.12	9.39	9.11	9.21±0.16	9.24	9.27	9.37	9.29±0.06	9.43	9.33	9.33	9.36±0.06	9.42	9.26	9.37	9.35±0.09
15	9.46	9.37	9.55	9.46±0.09	9.60	9.56	9.58	9.58±0.02*	9.44	9.30	9.36	9.37±0.07	9.30	9.33	9.32	9.32±0.02*
21	9.47	9.33	9.36	9.39±0.07	9.40	9.25	9.28	9.31±0.08	9.36	9.03	9.40	9.26±0.20	9.30	9.01	9.39	9.24±0.20
28	9.33	9.17	9.46	9.32±0.15	9.30	9.15	9.39	9.28±0.12	9.33	9.03	9.38	9.25±0.19	9.40	9.17	9.35	9.31±0.12
42	9.24	9.35	9.29	9.29±0.06	9.45	9.22	9.25	9.31±0.12	9.16	9.20	9.35	9.24±0.10	9.26	9.19	9.35	9.27±0.08
56	9.39	9.41	9.45	9.42±0.03*	9.48	9.35	9.49	9.44±0.08*	9.28	9.32	9.28	9.29±0.02*	9.31	9.26	9.21	9.26±0.05*

*Average values were statistically different between 20°C and 30°C fermentations according to Z-test.

doi:10.1371/journal.pone.0095449.t001

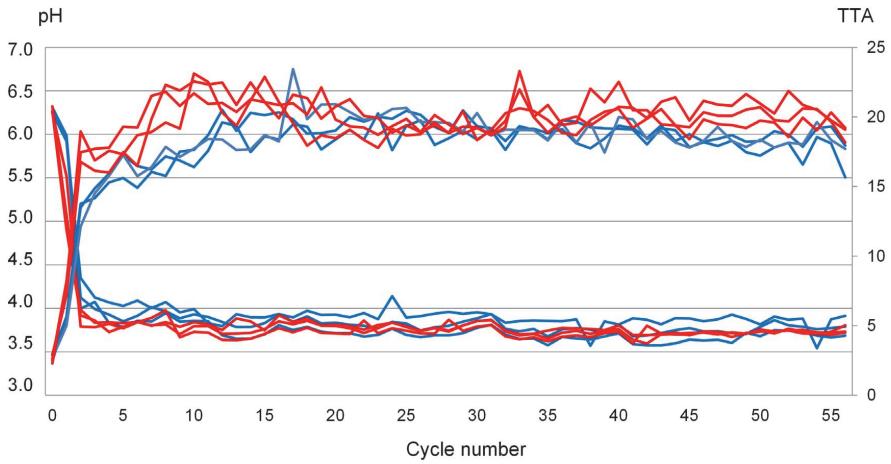

Figure 1. pH and total titratable acidity of six rye sourdoughs propagated at 20°C (20-I, 20-II and 20-III, shown in blue) and 30°C (30-I, 30-II and 30-III, shown in red) during 56 days.
doi:10.1371/journal.pone.0095449.g001

Dynamics of the Bacterial Communities Determined by Pyrosequencing of Bar-coded 16S rRNA Gene Amplicons

Pyrosequencing of bar-coded 16S rRNA gene amplicons was applied to overcome limitation of DGGE analysis and study in-depth the establishment of microbial consortia in spontaneously started rye sourdoughs. Widely used V1–V2 region specific primers were chosen to distinguish between different LAB species [24–26]. After matching the barcodes and performing initial quality processing using PyroNoise, 48912 raw reads were found. After removing 1258 chimers and plant chloroplast related sequences, a total of 41819 high-quality partial 16S rRNA gene sequences longer than 150 bp were used in the analysis (Table S1). The majority of high-quality reads were in the length range 280–310 bp. The number of detected OTUs, normalized number of expected OTUs at 500 sequences, and the rate of new OTUs when one sequence is added to the sample at 500 sequences are provided in Table S1. The latter quantity indicates that the sequence coverage was sufficient for most of the samples, with the exception of four (0–20-I, 1–20-II, 1–30-I, 5–30-III), which all had a lower number of reads (≤500). During the first three propagation cycles, species diversity was higher in sourdoughs fermented at 30°C as determined by the normalized number of expected OTUs (Table S1). This number of expected OTUs per 500 reads declined during the continuous propagation of the sourdoughs to between 4 and 9 after 56 renewals (Table S1).

Several bacterial species were found in the rye flour (Table S2), although the ratio of microbial DNA to rye DNA in this sample was low. As a consequence, only 60 high-quality bacterial reads were obtained. The main OTUs identified were from the genera *Pantoea* (33%) and *Stenotrophomonas* (15%) (Table S2). LAB species were represented by *Lactobacillus iners* (3.3%), *Leuconostoc citreum* (1.7%), and *Weissella cibaria* (1.7%).

After 24 hours of spontaneous flour fermentation at 20°C between 70–90% of all identified 16S rRNA gene amplicons were from the genera *Enterobacter*, and *Pantoea* (Table S2). Bacteria from the genera *Weissella*, *Leuconostoc*, *Lactococcus*, and *Lactobacillus* together comprised up to 23% of the bacterial community. In sourdoughs fermented at 30°C, the majority of the bacterial community was formed by representatives of the genera *Enterobacter*, *Weissella*, *Lactococcus*, and *Leuconostoc*. In contrast with the DGGE analysis, differences between the bacterial composition of sourdoughs fermented under the same conditions were already observed after the first fermentation cycle (Figure 3).

In sourdoughs fermented at 20°C, enterobacteria were totally replaced by the LAB species Weissella cibaria, Weissella paramesenteroides/confusa, Lactococcus lactis, Leuconostoc citreum, and Lactobacillus graminis/sakei/curvatus after the third renewal cycle on day 3. W. paramesenteroides/confusa and Lc. lactis formed the dominant population in sourdoughs 20-II and 20-III. In batch 20-I, W. paramesenteroides/confusa and L. graminis/sakei/curvatus dominated. The proportion of L. graminis/sakei/curvatus increased in all sourdoughs fermented at 20°C after the fifth fermentation cycle. This species kept its dominant position in batches 20-I and 20-II up to day 8 and formed over 80% of the bacterial community. In contrast, L. plantarum/pentosus formed over 70% of the bacterial community in sourdough 20-III. After day 21, L. plantarum and L. brevis were the dominant species in sourdoughs 20-I and 20-III, while in sourdough 20-II L. crustorum and L. plantarum formed 85% and 13% of identified amplicons, respectively. Even after 56 days of fermentation the dominant community in all three sourdoughs fermented at 20°C differed. In sourdough 20-I, Lactobacillus paralimentarius/kimchii (the latter is the synonym for L. paralimentarius [27]) and L. brevis dominated in the proportion 81:19. In sourdough 20-II the dominant community was represented by L. crustorum and L. brevis in the proportion of 46:50. In sourdough 20-III L. plantarum and L. brevis were dominated in the proportion 34:65.

In contrast with sourdoughs fermented at 20°C, enterobacteria were still present in low numbers within sourdoughs fermented at 30°C after the third renewal cycle. Various combinations of *W. paramesenteroides/confusa*, *Pediococcus pentosaceus*, *Leuc. citreum*, and *L. graminis/sakei/curvatus* were found in sourdoughs 30-I, 30-II, and 30-III. After the fifth renewal cycle, the dominant bacteria in 30-I and 30-II were *P. pentosaceus* and *L. plantarum/pentosus*, respectively. In sourdough 30-III, *L. plantarum*, *L. graminis/sakei/curvatus*, and *Lactococcus lactis* species were found in equal proportions (Figure 3).

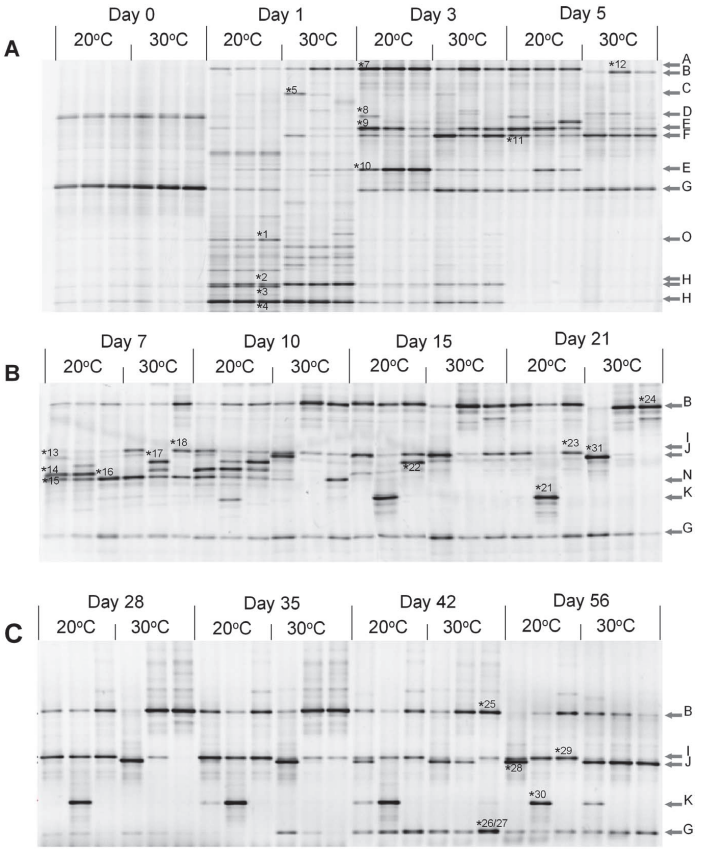

Figure 2. DGGE analysis of six rye sourdoughs propagated at 20°C (20-I, 20-II and 20-III) and 30°C (30-I, 30-II and 30-III) during 56 days. The arrows indicate specific bands for the following groups: (A) *Weissella* sp., (B) *Lactobacillus plantarum*, (C) *Enterobacteriaceae*, (D) *Lactobacillus* sp., (E) *Lactobacillus* sp., (F) *Pediococcus* sp., (G) Cereal chloroplast, (H) *Pantoea agglomerans*, (I) *Lactobacillus brevis*, (J) *Lactobacillus paralimentarius*, (K) *Lactobacillus crustorum*, (M) *Lactobacillus curvatus/graminis/sakei*, (N) *Pediococcus pentosaceus*, (O) *Gamma Proteobacteria* species. The bands marked by numbers were cut and sequenced. The closest matches are shown in the Table 2. A. DNA samples from days 0, 1, 3 and 5 of backslopping. B. DNA samples from days 7, 10, 15 and 21 of backslopping. C. DNA samples from days 28, 35, 42 and 56 of backslopping.
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On day 7, over 70% of the bacterial community in sourdoughs 30-I, 30-II, and 30-III belonged to either *L. brevis*, *L. plantarum/pentosus*, or *L. graminis/sakei/curvatus*, respectively. However, on day 21 further changes in the composition of sourdoughs 30-I and 30-III were detected. Over 90% of the bacterial amplicons identified in sourdough 30-I belonged to *L. paralimentarius/kimchii*, while *L. plantarum* dominated in sourdoughs 30-II and 30-III. On day 56, *L. paralimentarius* was the dominant species (>70%) in all sourdoughs fermented at 30°C, while the proportion of *L. plantarum* and *L. brevis* remained below 7%. In sourdough 30-I, *L. crustorum* was also found to comprise 15% of the bacterial community after 56 backslopping cycles.

Isolation, Identification, and Characterization of the Dominant Bacteria after 56 Backslopping Propagations

In order to obtain pure cultures of bacteria after 56 days of propagation we randomly picked 10 colonies from MRS agar and 10 from SDB agar for each of the six samples. In total, 120 colonies were selected and analyzed by rep-PCR fingerprinting. Four different Rep-PCR fingerprint groups were detected (data not shown). Representatives of each group were identified using 16S rRNA partial gene sequencing and were found to be *L. plantarum* (*L. plantarum* M30I-1, GenBank accession number KJ361844), *L. brevis* (*L. brevis* M30I-2, GenBank accession number KJ361843), *L. paralimentarius* (*L. paralimentarius* M30I-3, Genbank accession number KJ361845) and *L. crustorum* (*L. crustorum* M30I-9, GenBank accession number KJ361846) with at least 98% identity. In most cases the fraction of each species identified in the sourdough samples are comparable with pyrosequencing data (Figure 4).

Metabolic profiles of the four dominant LAB species were determined (Table 3). The strain *L. plantarum* M30I-1 was able to ferment the largest range of carbohydrates. Together with *L. paralimentarius* M30I-3, *L. plantarum* M30I-1 was able to ferment all

Table 2. Identification of DGGE bands obtained from six rye sourdoughs propagated at 20°C and 30°C during 56 days.

Band number on DGGE	Closest match in GenBank	Similarity	Accession Nr.
1	*Gamma Proteobacteria* species	163/168 (97%)	gb|GU352675.1
2	*Pantoea agglomerans*	166/166 (100%)	gb|KC355300.1
3	*Pantoea agglomerans*	165/166 (99%)	gb|KC355300.1
4	*Pantoea agglomerans*	165/166 (99%)	gb|KC355300.1
5	*Enterococcus casseliflavus*	120/140 (86%)	ref|NR_102793.1
7	*Weissella confusa*	160/168 (95%)	gb|KC845208.1
8	*Lactobacillus graminis*	97/106 (92%)	gb|KC836565.1
9	*Lactobacillus curvatus*	133/155 (86%)	gb|FJ609221.1
10	*Lactobacillus curvatus*	143/155 (92%)	gb|FJ609221.1
11	*Pediococcus acidilactici*	118/124 (95%)	gb|JF268323.1
12	*Lactobacillus plantarum*	138/138 (100%)	gb|JN863682.1
13	*Lactobacillus curvatus*	247/250 (99%)	gb|KF411435.1
13	*Lactobacillus graminis*	247/250 (99%)	gb|KF149819.1
13	*Lactobacillus sakei*	247/250 (99%)	gb|KF149680.1
14	*Lactobacillus curvatus*	206/217 (95%)	gb|KF411435.1
14	*Lactobacillus graminis*	206/217 (95%)	gb|KF149819.1
14	*Lactobacillus sakei*	206/217 (95%)	gb|KF149680.1
15	*Pediococcus pentosaceus*	276/276 (100%)	gb|JN851781.1
16	*Pediococcus pentosaceus*	254/257 (99%)	gb|JN851779.1
17	*Lactobacillus sakei*	276/276 (100%)	gb|JN851763.1
18	*Lactobacillus brevis*	257/257 (100%)	gb|JN863690.1
21	*Lactobacillus crustorum*	258/261 (99%)	gb|KF193907.1
23	*Lactobacillus brevis*	132/132 (100%)	gb|KC845206.1
24	*Lactobacillus plantarum*	140/140 (100%)	gb|JN863682.1
25	*Lactobacillus plantarum*	259/259 (100%)	gb|KF318862.1
26	*Secale cereale, complete genome*	121/122 (99%)	gb|KC912691.1
27	*Secale cereale, complete genome*	120/122 (99%)	gb|KC912691.1
28	*Lactobacillus paralimentarius*	113/113 (100%)	gb|KC755102.1
29	*Lactobacillus brevis*	129/129 (100%)	gb|KC845206.1
30	*Lactobacillus crustorum*	113/113 (100%)	gb|KC755094.1
31	*Lactobacillus paralimentarius*	114/114 (100%)	gb|KC755102.1

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four major cereal carbohydrates (glucose, fructose, maltose, saccharose) while *L. brevis* M30I-2 and *L. crustorum* M30I-9 were able to ferment either glucose and fructose or glucose, fructose, and maltose, respectively.

Isolation, Identification, and Characterization of the Dominant Yeasts after 56 Backslopping Propagations

After 56 days of propagation 10 colonies were picked from YPD agar plates for each sourdough sample (60 colonies in total) and fingerprinted using M13 primer. Four different patterns were observed (data not shown) and representatives from each group were identified. They belong to *Kazachstania unispora* (*K. unispora* Y30I-10, GenBank accession number KJ361847), *Candida glabrata* (*C. glabrata* Y30II-1, GenBank accession number KJ361850), *Saccharomyces cerevisiae* (*S. cerevisiae* Y30II-9, GenBank accession number KJ361848) or *Candida krusei* (*C. krusei* Y30II-5, GenBank accession number KJ361849) based on 26S rRNA partial gene sequencing. *K. unispora* was found to be the only dominant species in all sourdoughs fermented at 20°C, but was also found in sourdough 30-I which was propagated at 30°C. *C. glabrata* was the only yeast species found in sourdough 30-III while sourdough 30-II contained three yeast species, *S. cerevisiae*, *C. krusei*, and *C. glabrata*, in the proportion 2:3:5.

Isolated representatives of *K. unispora*, *C. krusei*, and *C. glabrata* were not able to ferment maltose and had a narrow carbohydrate fermentation profile compared with *S. cerevisiae* (Table 4). However, only *C. krusei* and *C. glabrata* could ferment *N*-acetylglucosamine and trehalose, respectively.

Discussion

Previous research has established that stabilization of LAB consortia in spontaneously started sourdoughs occurs in a three-stage evolution process over the course of five to ten days [2,8,10–11,28]. During this time the acidity drops and stabilizes together with the LAB count to a level common in mature sourdough. The majority of these studies used liquid sourdoughs, which were fermented using continuous stirring. To our knowledge there are

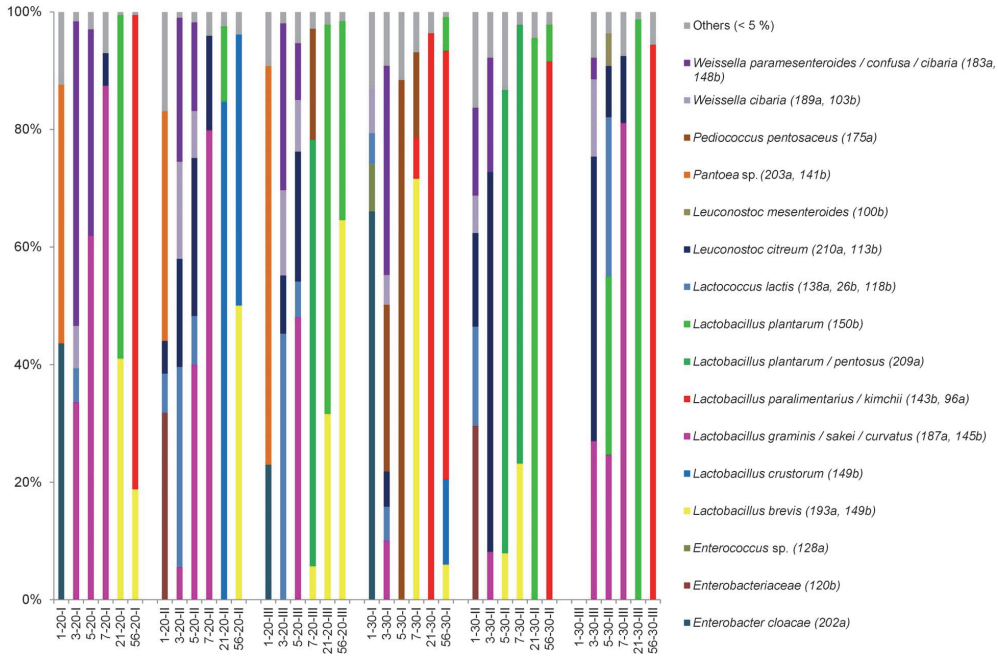

Figure 3. Pyrosequencing of 16S rRNA gene amplicons from spontaneous rye sourdoughs propagated for 56 days. Three sourdoughs were fermented at 20°C (20-I, 20-II, 20-III) and three sourdoughs fermented at 30°C (30-I, 30-II, 30-III). Sourdoughs were sampled at days 1, 3, 5, 7, 21, 56. The relative abundance at the species level based on partial 16S rRNA gene sequences is given. Species forming less than 5% of the population were grouped together and are shown as 'Others (<5%)'. (a) and (b) stand for two different sequencing runs.
doi:10.1371/journal.pone.0095449.g003

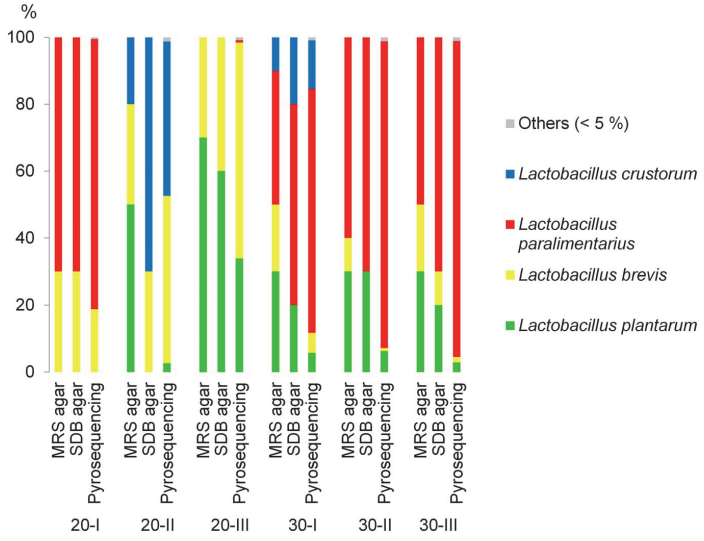

Figure 4. Ratio of species in the sourdoughs fermented at 20°C (20-I, 20-II, 20-III) or 30°C (30-I, 30-II, 30-III) after 56 backslopping cycles determined by plating on MRS and SDB media or by pyrosequencing of 16S rRNA gene amplicons.
doi:10.1371/journal.pone.0095449.g004

Table 3. Carbohydrate fermentation profiles of *Lactobacillus* species isolated on day 56 of sourdough backlopping.

Active ingredient	*Lactobacillus plantarum* M30I-1	*Lactobacillus brevis* M30I-2	*Lactobacillus paralimentarius* M30I-3	*Lactobacillus crustorum* M30I-9
L-arabinose	+	+	+	−
D-ribose	+	+	+	−
D-xylose	+	+	+	−
D-galactose	+	+	−	+
D-glucose	+	+	+	+
D-fructose	+	+	+	+
D-mannose	+	−	+	+
D-mannitol	+	−	−	−
D-sorbitol	+	−	−	−
N-acetylglucosamine	+	+	+	+
Amygdalin	+	−	+	+
Arbutin	+	−	+	−
Esculin ferric citrate	+	−	+	−
Salicin	+	−	+	+
D-celiobinose	+	−	+	+
D-maltose	+	+	+	+
D-lactose (bovine origin)	+	−	−	+
D-melibiose	+	+	−	−
D-saccharose (sucrose)	+	−	+	−
D-trehalose	+	−	+	+
Inulin	+	−	−	−
D-melezitose	+	−	+	−
D-raffinose	+	−	−	−
Gentiobinose	+	−	+	+
Sodium Gluconate	+	+	+	−
Sodium 5-Ketogluconate	−	+	−	−

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no studies that have monitored the fate of a bacterial community after the sourdough has reached maturity under controlled laboratory conditions, where the raw flour is the sole bacterial source.

We followed 56 daily backslopping cycles of spontaneously started semi-solid rye sourdough at two temperatures with six parallels. While the sourdoughs achieved maturity in 10 to 12

Table 4. Carbohydrate fermentation profiles of yeast species isolated on day 56 of sourdough backlopping.

Active ingredient	*Saccharomyces cerevisiae* Y30II-9	*Kazachstania unispora* Y30I-10	*Candida krusei* Y30II-5	*Candida glabrata* Y30II-1
D-glucose	+	+	+	+
Glycerol	−	−	+	−
D-galactose	+	+	−	−
N-acetylglucosamine	−	−	+	−
D-maltose	+	−	−	−
D-saccharose (sucrose)	+	−	−	−
D-trehalose	−	−	−	+
D-melezitose	+	−	−	−
D-raffinose	+	−	−	−

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propagation cycles, further succession of LAB species was observed even after 42 cycles.

The low number of sourdough specific LAB in rye flour could be the main reason for the observed instability. The concentration of bacteria in rye flour varies between 10^4 to 10^6 CFU/g depending on the climate, time of harvest, and both milling and storage conditions [29]. Using 16S rRNA gene pyrosequencing we found that the microbiota in the raw flour used in this study contained LAB species at a subdominant level (<7%) and the majority of these were species that typically do not dominate in sourdough communities. Thus the strains that dominated in the six mature sourdough parallels may have been present in very low concentrations in the raw flour. The effect of introducing low numbers of sourdough competent LAB together with the small volumes of flour used to prepare the sourdoughs could together work to create an uneven distribution of bacteria between the three parallels conducted at the same temperature. As with our observations, Minervini et al. [31] observed the succession of LAB strains during propagation by comparatively following the microbial community of mature wheat sourdough during propagation in an artisan bakery and a controlled laboratory environment. They attribute the cause of the observed succession to be differences between the batches of flour used.

The temperature used for sourdough fermentation is one of the key factors determining the composition of the microbial community in sourdough [15,32]. The process of establishing sourdough microbial consortia occurred more rapidly at 30°C, as evidenced by the prevalence of LAB in these sourdoughs already after the first fermentation cycle. Several studies have shown that only one fermentation at 30°C is needed to enrich LAB in sourdough [11,32], whereas up to three renewal cycles were needed to detect LAB at a lower temperature (23°C) [32]. We also observed LAB after one fermentation at 20°C, albeit at a significantly lower fraction of the total community compared with fermentations at 30°C. In addition, the normalized number of expected OTUs indicates that the species richness during the first three propagation cycles was higher in sourdoughs fermented at higher temperature. Interestingly, enterobacteria persisted over more fermentation cycles in bacterial communities of sourdough fermented at 30°C (up to three fermentations) than in sourdoughs fermented at 20°C.

Facultative heterofermentative bacteria dominated in sourdoughs fermented at 30°C after 56 propagation cycles, while in sourdoughs fermented at 20°C both obligate and facultative heterofermentative LAB were dominant. It has been shown that despite the metabolic inefficiency of obligatory heterofermentative lactic acid bacteria, these species commonly dominate in sourdough fermentations [2]. Balance between homofermentative and heterofermentative lactic acid bacteria depends on dough yield, redox potential and fermentation temperature [30]. In this study fermentation temperature shifted the ratios between the limited number of LAB species in the community rather than select for different species. Rep-PCR analysis of isolates obtained from the sourdoughs after 56 renewal cycles revealed that fingerprints among isolates of the same species were similar.

Pyrosequencing results establish that during the development of bacterial consortia in sourdough, irrespective of fermentation temperature, *L. plantarum* dominated or codominated in the majority of sourdoughs. However, after 56 propagation cycles *L. plantarum* had been replaced by *L. paralimentarius* in most batches. *L. plantarum* is considered a highly acid-tolerant LAB that dominates in fermentation processes with vegetables and cereals due to its metabolic flexibility and low pH adaptation [33–35]. It also dominated in bacterial communities of four liquid spontaneously started laboratory rye sourdoughs after 10 backslopping renewals [8]. The carbohydrate fermentation pattern of *L. paralimentarius* isolated in this study is more constrained compared with *L. plantarum*, however, they are able to ferment all major carbohydrates (maltose, saccharose, fructose, and glucose) [36]. On the other hand, succession within bacterial communities may depend on many other factors aside from carbohydrate metabolism, including amino acid metabolism and tolerance to acid stress conditions [10]. Several studies have shown that the competiveness of LAB often depends on their intraspecies diversity and is strain-specific [28,36].

Fermentation temperature also influenced the composition and diversity of the yeast community within the sourdoughs studied. Both yeast species detected in 30°C fermentations after 56 propagation cycles, i.e. *Saccharomyces cerevisiae* and *Candida krusei* belong to the six most frequently encountered species in sourdoughs [37], while *Candida glabrata* is considered to be a prevailing species during liquid laboratory sourdough and teff fermentations [28,38]. *Kazachstania unispora* was the only yeast species found in sourdoughs fermented at 20°C. This species has been documented twice to exist in a sourdough ecosystem, albeit in low abundance: once in Belgian artisan wheat sourdough [37] and once in Finnish rye sourdough starter [39]. In contrast, *K. unispora* plays a significant role in both the ripening of cheese and in the production of fermented milk products such as a kefir and koumiss (reviewed by [40]). Additionally orange, sugarcane or mixed vegetable juices favour growth of *K. unispora*. Ambient or low temperatures and high organic acid concentrations are specific for most of these processes [38–39,41–44]. No clear relationship was found between the yeast and LAB strains detected in this study. However, most isolated yeast species were maltose negative and trophic interactions between LAB and yeasts could be suspected because cooperation in utilization of maltose is the most frequently cited reason for the co-occurrence of yeasts and LAB (reviewed in [45]).

Studies of microbial consortia in food matrices, such as sourdoughs, are commonly based on a combination of culture dependent and culture independent methods [46–47]. This study applies both approaches to analyze the bacterial community within mature sourdoughs after 56 propagation cycles. Plating on both SDB and MRS media, which are both commonly used for sourdough specific LAB isolation [48], provided similar numbers of colony forming units. In most samples species occurrence and ratio among identified isolates was comparable with pyrosequencing data.

The results of both DGGE and 16S rRNA gene pyrosequencing were generally in agreement despite the fact that they amplified different regions of the 16S rRNA. The bacterial diversity suggested by pyrosequencing results was greater than that revealed by DGGE because of the differences in their detection limits. Both methods are based on DNA amplification and share similar limitations, e.g. possible inefficient DNA isolation from some organisms. However, high-throughput sequencing analysis is considered quantitative if efforts are made to minimize changes in the original proportion of microbial cells caused by DNA extraction [11]. Amplification of DNA from dead organisms could be critical for the first step of sourdough propagation which would overestimate the diversity of metabolically active bacteria in flour and sourdoughs. Similar distribution of OTUs between DNA and RNA samples has been shown for mature sourdoughs [11].

Both DGGE and pyrosequencing often fail to discriminate between closely related species due to both the insufficient length and accuracy of sequences. Current developments in high-throughput sequencing technologies are expected to overcome

these problems. Despite its limitations, high-throughput sequencing has the potential to become a powerful tool for the culture-independent study of sourdough microflora since it offers a more in-depth analysis [49].

Supporting Information

Table S1 Characterization of pyrosequencing data obtained from the analysis of sourdough samples.
(PDF)

Table S2 Relative abundance (%) of partial 16S rRNA gene sequences obtained by pyrosequencing of spontaneous rye sourdoughs propagated for 56 days.
(PDF)

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Author Contributions

Conceived and designed the experiments: EV MB IS TP. Performed the experiments: EV MB. Analyzed the data: EV MB IS JS. Contributed reagents/materials/analysis tools: EV MB JS TP IS. Wrote the paper: EV MB IS TP JS.

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Publication IV

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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

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

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.

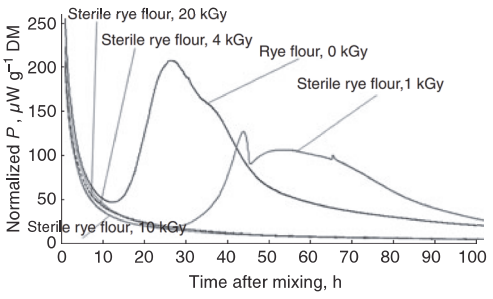

Figure 1 The power–time curves of rye dough prepared from nonirradiated (0 kGy), 1, 4, 10 and 20 kGy irradiated flour.

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.

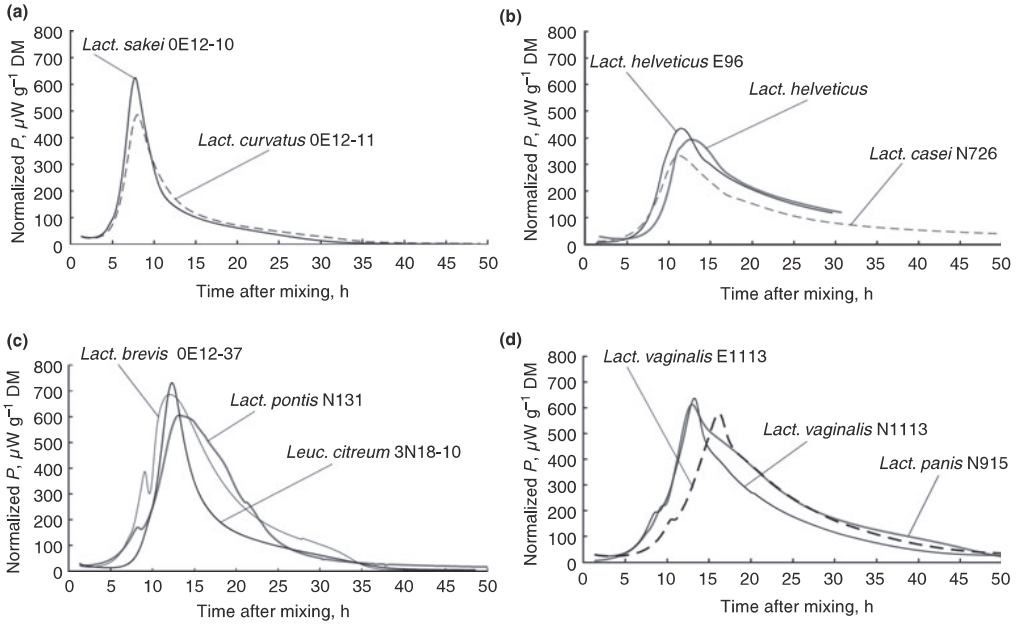

Figure 2 Power–time curves of sourdough samples inoculated with the different homofermentative (a,b) and heterofermentative (c,d) strains of LAB.

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

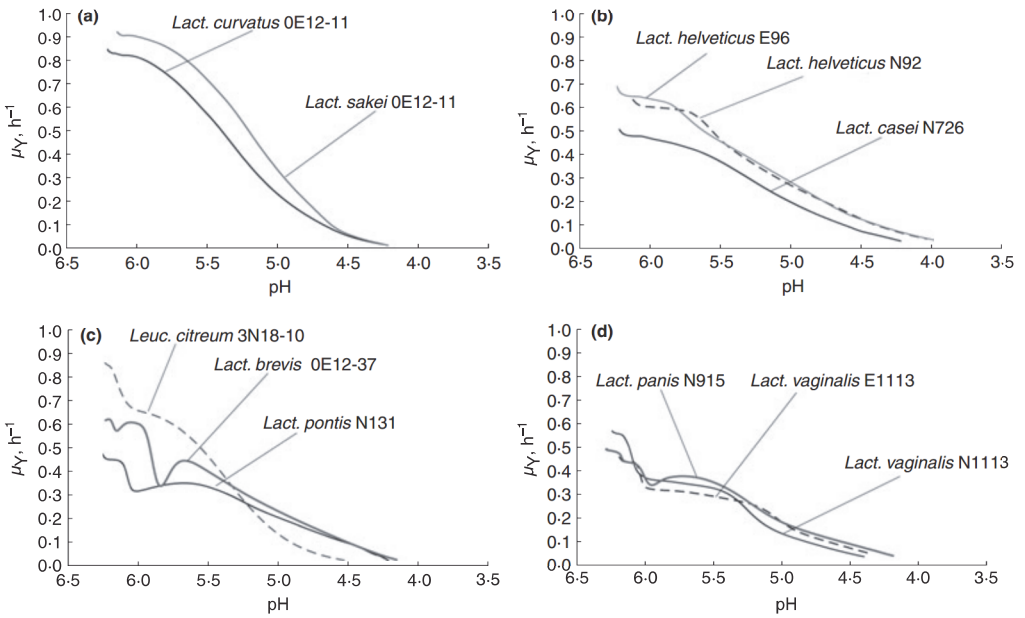

Figure 3 The dependence of LAB specific growth rate in sourdough from pH. (a,b) – homofermentative species, (c,d) heterofermentative species.

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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