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Selection of Functional Starter Bacteria for Type I Sourdough Process

MARIANNA SURŽENKO

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TALLINN UNIVERSITY OF TECHNOLOGY
Department of Chemistry and Biotechnology

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Supervisor: Doctor Inga Sarand
Department of Chemistry and Biotechnology
Tallinn University of Technology (TUT)

Opponents: Doctor Delphine Sicard
Research Director
French National Institute for Agricultural Research, France

Professor Gražina Juodeikiene
Department of Food Science and Technology
Kaunas University of Technology, Lithuania

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Declaration:

Hereby I declare that this doctoral thesis, submitted for the doctoral degree at TUT, is my original investigation and achievement and has not been submitted for the defence of any other academic degree elsewhere.

Marianna Surženko

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LIST OF PUBLICATIONS

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I. Viiard, E., **Bessmeltseva, M**., Simm, J., Talve, T., Aaspõllu, A., Paalme, T and Sarand, I. (2016) Diversity and stability of lactic acid bacteria in rye sourdoughs of four bakeries with different propagation parameters. *PLos ONE* 11(2): e0148325. Doi:10.1371/journal.pone.0148325

II. **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

III. **Surženko, M**., Part, N., Rosend, J., Kelman, E., Kontram, K and Sarand, I. (2017) Characterization of lactic acid bacteria isolated from spontaneously started rye sourdough and their potential application as starter bacteria for Type I sourdough production. (Unpublished manuscript).

IV. **Surženko, M**., Kontram, K and Sarand, I. (2017) PCR–based fingerprinting and identification of contaminative fungi isolated from rye breads. *Agronomy Research* 15(1): 288–297.

AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

In Publication I, the author isolated and identified lactic acid bacteria and yeasts from sourdoughs, performed the DGGE analysis of sourdoughs yeast populations and participated in the writing the manuscript.

In Publication II, the author performed the experimental work, isolated and identified bacteria and fungi, performed DGGE analysis, interpreted the data, and wrote the manuscript.

In Publication III, the author planned and performed experimental work, interpreted the data, and wrote the manuscript.

In Publication IV, the author planned the experimental work, isolated DNA from fungi, performed amplification of targeted genome regions, interpreted the data, and wrote the manuscript.

LIST OF CONFERENCE PRESENTATIONS

I. 5th Baltic Conference of Food Science and Technology, FoodBalt 2010, October 2010, Tallinn, Estonia. "Properties and microbial population stability of model rye sourdough started with bacteria isolated from industrial rye sourdough" *(Poster presentation)*

II. The 10th Symposium on Lactic Acid Bacteria, September 2011, Egmond aan Zee, the Netherlands. "Temperature effect on population dynamics of lactic acid bacteria during 56–day long spontaneously initiated rye sourdough processes" *(Poster presentation)*

III. FoodMicro 2012, September 2012, Istanbul, Turkey. "Spontaneous Fermentation of Sourdough as Natural selection of Highly adapted Starter Bacteria" *(Poster presentation)*

IV. Cereal Fermentation for Future Foods 2012, October 2012, Helsinki, Finland "Evaluation of Yeast Populations in Spontaneously Fermented Rye Sourdoughs Using Different Cultural–Independent Methods" *(Poster presentation).* Best poster award

V. 1st Symposium of Baltic Microbiologists, November 2012, Riga, Latvia. "CCFFT – Emergency for Small–scale Bakeries" *(Oral presentation)*

VI. The 5th Congress of European Microbiologists, July 2013, Leipzig, Germany. "Metagenomic detection of yeast biodiversity in the cereal and dairy based foods" *(Oral and Poster presentation)*

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INTRODUCTION

Sourdough can be described as a biologically active microbial preparation made from a mixture of flour and water fermented by lactic acid bacteria (LAB) and yeasts (Vogel et al., 1999). Sourdough is an essential intermediate product for the production of traditional rye bread in Estonia and other northeastern European countries (Vajakka, Kerojoki and Katina, 2003). Rye flours characteristically have active amylolytic enzymes and a weak ability to form a gluten matrix, thus making rye dough sensitive to the acidification rate (Stolz, 2003; De Vuyst and Neysens, 2005). The metabolites produced by lactic acid bacteria during sourdough fermentation both improve the technological properties of the dough and also contribute to an increase in dough volume and the formation of desirable sensory properties of rye bread (Gobetti et al., 1995). Moreover, fermentation of the dough play a crucial role in bioprotection of the bread (Lavermiocca et al., 2000; De Vuyst and Neysens, 2005; Gerez et al., 2009). As a result, fermentation failure often leads to significant economic losses for baking facilities due to both fluctuations in texture and organoleptic qualities and a reduced shelf-life of their baked products.

Controlled fermentation conditions, together with high propagation temperatures, are utilized in most large–scale bakeries to stabilize sourdough microbiota and thereby ensure that they consistently produce high–quality end products (Vogel et al., 1996; De Vuyst and Neysens, 2005). However, many artisan bakeries still use traditional sourdough propagation technologies and face sufficient season–dependent fluctuations in microbial composition, which is largely caused by unstable fermentation conditions (De Vuyst and Neysens, 2005; Minervini et al., 2014).

When well–controlled production is impossible, the use of starter bacteria that is highly adapted to that specific sourdough type and ambient fermentation temperatures appears to be the most rational way to consistently produce high-quality end products. Yet, the selection of starter bacteria for the baking industry is typically made based on the specific technological properties of the strain such as fast acidification or the production of desirable volatile compounds. This approach underestimates the importance of the adaptation of potential starter bacteria to the flour type and specific technological parameters applied in the current bakery (Leroy and De Vuyst, 2004; Coda et al., 2014).

The main aim of this work was to select a robust, metabolically active and functional LAB starter for the further use in Type I sourdough processes. For this we (i) determined which technological parameters critically affect the stability of industrial Type I rye sourdoughs; (ii) applied these as selective factors in evolution experiments to find robust LAB strains; and (iii) isolated and characterized dominant LAB strains. In addition, biodiversity of fungi responsible for microbiological spoilage of rye bread was evaluated.This work continues the studies of Ene Viiard who focused on the diversity and stability of lactic acid bacteria during rye sourdough propagation.

ABBREVIATIONS

AGE – agarose gel electrophoresis

ATP – adenosine triphosphate

CFS – cell–free supernatant

CFU – colony forming unit

DGGE – denaturing gradient gel electrophoresis

DM – dry matter

DNA – deoxyribonucleic acid

DY – dough yield

EMP – Emden–Meyerhof–Parnas pathway

GRAS – Generally Regarded as Safe

HPLC – high–performance liquid chromatography

IMC – isothermal microcalorimetry

ITS – internal transcribed spacer

kGy – kiloGrey

LAB – lactic acid bacteria

MRS – de Man, Rogosa and Sharpe medium

μmax – maximum specific growth rate

NADH – nicotinamide adenine dinucleotide hydride

OTU – operational taxonomic unit

PCR – polymerase chain reaction

P_{max} – maximum heat flow

PTS – phosphotransferase system

RAPD – Random amplified polymorphic DNA analysis

rRNA – ribosomal ribonucleic acid

SDB – SourDough Bacteria medium

SPME – solid–phase microextraction

STDEV – standard deviation

TAMIII – Thermal Activity Monitor III

t_{Pmax} – time at maximum heat production rate

TTA – total titratable acidity

YPD – yeast extract peptone dextrose medium

6–PG/PK – 6–phosphogluconate/phosphoketolase

1. LITERATURE REVIEW

1.1 Traditional rye bread in Estonia

Rye bread could be defined as bread prepared using at least 90% rye flour (Kapten–Leppik, 2007). Rye bread is traditionally consumed at a very high level in Estonia. According to Valjakka, Kerojoki and Katina (2003) Estonians eat approximately 19.9 kg of rye bread yearly. Rye bread is made without the addition of fat or milk, is rich in vitamins and dietary fibers, and is considered a valuable part of our daily diet (Liukkonen et al., 2007).

As with any other Baltic country, Estonia has its own traditions of breadmaking. Traditional Estonian rye bread could be characterized by a specific sweet sour taste – the result of dough fermentation and the addition of scalded rye flour. The scalding step not only allowed one to inactivate undesirable enzymes of sprouted rye, but also sterilized the flour and thus stabilized the substrate used in fermentation (Valjakka, Kerojoki and Katina, 2003). Sourdough used for the initiation of fermentation process was typically kept in wooden kegs and was added to the cooled cooked flour. When the sourdough ripening process ended, the remaining flour was added and mixed into dough. Currently, many modern bakeries use sugar syrups in order to increase the sweetness of the dough either together with scalded flour or as a complete substitution of the scalding step. Caraway seeds that are either added to the dough during the sourdough fermentation process or applied directly to the surface of the bread loaf for decoration can also be considered as features of a traditional rye bread produced in the Baltic region (Valjakka,Kerojoki and Katina, 2003).

Nowadays, many countries have replaced the traditional bread leavening process by adding baker's yeasts or food additives. However, the sourdough process remains as a keystone of rye breadmaking in the Baltics. Moreover, new trends in healthy, preservative–free products started to turn customers back towards small local bakeries. Despite the fact that sliced and packed rye bread produced in large scale bakeries still dominate total sales, the popularity of small–scale bakeries with their "green", baker's yeasts–free, handmade, rye breads is rapidly growing (Valjakka, Kerojoki and Katina, 2003; Joudeikiene, 2016). Also, the application of starter cultures simplifies the handling of sourdough in the production of traditional sourdough rye breads in artisanal bakeries (Stolz, 2003).

1.2 Acidification of the dough as essential part of rye bread production

Rye flour has remarkably different baking properties compared to wheat flour. The structure of the crumb in bread prepared from wheat flour is based on the gluten matrix and consists of proteins including gliadin and glutenin. The gluten matrix is crucial for the gas holding capacity of wheat dough and therefore influences the volume of the bread. In contrast, rye proteins cannot form a proper gluten matrix because the gliadin and glutenin fractions are lacking in rye (Stolz, 2003; De Vuyst and Neysens 2005). Instead, both the structure of the crumb and the gas retention capacity of the rye dough completely depend on the ability of rye pentosans to bind water (Liukkonen et al., 2007). In order to improve the water binding capacity of rye pentosans it is recommended to decrease the pH of the dough to 4.4 – 4.2. Low pH also promotes peptisation and swelling of rye proteins which improves the rheology of the dough (Martinez–Anaya and Devesa, 2000).

Another important difference between wheat and rye flour is the presence of active α–amylase in the latter. The optimal activity of α–amylase in rye flour lies in the same range as gelatinization temperature of rye starch (53 – 64°C). Thus, doughs that containin more than 20% rye flour should be acidified to inhibit excessive starch degradation caused by the action of α–amylases. High acidity decreases the inactivation temperature of α–amylases, and thereby shortens the period of dextrin production and decreases the viscidity of the crumb (Corsetti and Settanni, 2007; Stolz, 2003; De Vuyst and Neysens, 2005). Taken together, dough acidification is essential for the production of high quality rye bread. A desirable pH of the dough can be achieved by adding chemical acidifiers, however, their use in food manufacturing is restricted by food legislation and rejected by consumers (Leroy and De Vuyst, 2004). It is likely that the modern baking industry can use sourdough as a natural alternative to chemical acidification of the dough.

1.3 Sourdough

Sourdough could be defined as a mixture of flour and water fermented by a microbial community of LAB and yeasts (De Vuyst and Neysens, 2005). Traditional rye sourdoughs are usually propagated by mixing a part of the ripped sourdough with fresh flour and water – a process which is known as “refreshing” or “backslopping” (Hansen, 2004; De Vuyst and Neysens, 2005). The bacterial community within mature rye sourdough is typically dominated by members from the genus *Lactobacillus* while yeasts from the genus *Candida* and *Saccharomyces* typically dominate (Gobbetti, Corsetti and Rossi, 1994; Stolz, 2003; De Vuyst et al., 2016; Gänzle and Ripari, 2016).

The main function of LAB in dough fermentation is the conversion of carbohydrates into lactic and acetic acids, while sourdough yeasts mainly

contribute to the formation of volatile compounds and increase the bread volume by their production of carbon dioxide (Stolz, 2003). Dissociated organic acids produced by sourdough LAB decrease the pH of rye dough and improve its rheology, while undissociated forms inhibit the growth of undesirable microflora (Stolz, Hammes and Vogel, 1996; Martinez–Anaya and Devesa, 2000; Lavermicocca, Valerio and Visconti, 2003). Besides lactic and acetic acids, LAB produce a range of other compounds that affect the structure, aroma, and color of bread. Among them ethanol, diacetyl, and acetaldehyde are the most important (De Vuyst and Neysens, 2005).

1.3.1 Carbohydrate metabolism of sourdough fermenting lactic acid bacteria

LAB associated with sourdough fermentation can be classified into three categories based on their metabolism:

I. Obligate homofermetative (e.g., *Lactobacillus amylovorus, Lactobacillus farciminis, Lactobacillus mindensis*) – LAB species which ferment hexoses via the Emden–Meyerhof–Parnas (EMP) pathway. These LAB mainly produce lactic acid and do not ferment pentoses due to the absence of the enzyme phosphoketolase (Axelsson, 1998; Kandler, 1983).

II Facultative heterofermentative (e.g., *Lactobacillus plantarum, Lactobacillus paralimentarius, Lactobacillus casei*) – LAB species which ferment hexoses via EMP pathway and possess the enzyme phosphoketolase which allows them to ferment pentoses via the 6–phosphogluconate/phosphoketolase (6–PG/PK) pathway (Kandler, 1983; Axelsson, 1998).

Both obligate homofermetative and facultative heterofermentative LAB preferentially transport carbohydrates via the phosphotransferase system (PTS). The metabolism of hexoses other than glucose is subjected to carbon catabolite repression and fructose is used (if used) only as a carbon source (Gänzle, 2015).

III. Obligate heterofermentative (e.g., *Lactobacillus sanfranciscensis, Lactobacillus pontis, Lactobacillus brevis*) – LAB species which ferment both pentoses and hexoses via 6–PG/PK pathway. Pentoses are fermented to lactic and acetic acid, whereas hexoses are converted either to lactic acid and ethanol or to acetic acid and carbon dioxide in equimolar amounts. Under anaerobic conditions, hexose degradation via the 6–PG/PK pathway results in only 1 mole of adenosine triphosphate (ATP) in contrast with the 2 moles produced as a result of homofermentative hexose degradation. However, many obligate heterofermentative LAB are able to gain additional energy by the activity of acetate kinase in the presence of external electron acceptors such as oxygen, fructose or citrate. This alternative pathway allows recycling of nicotinamide adenine dinucleotide hydride (NADH) and results in the production of acetate instead of ethanol and an additional mole of ATP (Kandler, 1983; Axelsson, 1998; De Vuyst and Neysens, 2005; Gänzle, 2015).

Obligate heterofermentative LAB species prefer disaccharides over glucose during fermentation. The PTS systems in this category of LAB are not functional and fructose is preferentially used as an electron acceptor (Gänzle, 2015).

Whereas homofermentative LAB prevail in a majority of food fermentations, in sourdough production, especially in traditional processes, heterofermentative LAB species dominate (Corsetti and Settanni, 2007; Gänzle and Ripari, 2016). The use of fructose as an external electron acceptor by heterofermentative LAB favors their competitiveness in a sourdough ecosystem. In addition, heterofermentative sourdough lactobacilli such as *Lb. sanfranciscensis, Lb. pontis,* and *Lb. brevis* possess maltose phosphorylase, a key enzyme responsible for the dominance of these LAB in sourdough. This enzyme allows for the phosphorolytic cleavage of maltose without using ATP during the phosphorylation process (Gänzle 2015). These species utilize maltose and accumulate glucose in the media in a molar ratio of about 1:1. Glucose liberated via maltose phosphorylase activity can be further used by maltose–negative LAB and yeasts (Kandler, 1983; Axelsson, 1998; De Vuyst and Neysens, 2005).

1.3.2 Classification of sourdoughs

On the basis of the technology applied, sourdoughs can be classified into three groups (Böcker, Stolz and Hammes, 1995):

– Type I or traditional sourdoughs
– Type II or accelerated sourdoughs
– Type III or freeze–dried sourdoughs

Type I sourdoughs, also called traditional sourdoughs, are characterized by a firm or semi–liquid consistency and are produced in long fermentations at ambient temperature (20 – 30°C). Regular propagation of the sourdough is applied in order to keep the sourdough microbiota in an active state. This type of sourdough is often dominated by obligately heterofermentative LAB such as *Lb. sanfranciscensis, Lb. brevis, Lb. pontis* and *Lactobacillus fermentum* in association with maltose–negative yeasts *Candida humilis*, *Candida holmii* or *Saccharomyces exiguus*. (De Vuyst and Neysens, 2005). Gänzle and Ripari (2016) reported that *Lb. sanfranciscensis* was isolated from more than 75% of 217 revived sourdoughs, hence making it the most frequently reported species in Type I sourdough. Depending on the fermentation conditions, other LAB species such as facultative heterofermentative *Lb. plantarum, Lb. paralimentarius, Lb. casei* and obligate homofermentative *Lb. mindensis, Lactobacillus acidophilus, Lactobacillus delbrueckii* may be present (Hammes and Gänzle, 1998; Vogel et al., 1999). Because both indigenous yeasts and heterofermentative LAB emit carbon dioxide during the fermentation, Type I sourdough could be applied for dough leavening without the additional use of baker's yeasts. The fermentation time of traditional sourdoughs depends on the technological scheme employed

within each bakery and may vary between 3 to 48 hours. The pH of a mature Type I sourdough is about 4.0 (De Vuyst and Neysens, 2005).

Together with the growing scales of the rye bread production, the need for more effective and highly controlled sourdough handling processes became more important. As a result, Type II sourdoughs were developed (De Vuyst and Neysens, 2005). Because of their liquid consistency, Type II sourdoughs can be pumped and thus easily transported within the production area. A liquid consistency also allows one to run large–scale fermentation process using thermostated bioreactors or tanks equipped with pH and temperature sensors (Decock and Capelle, 2005). The duration of a typical Type II sourdough fermentation process is longer than those applied for traditional sourdoughs and usually lasts between 2 – 5 days. In order to accelerate the process of acidification, higher fermentation temperatures (>30°C) are applied. The acidity of Type II sourdoughs is higher than traditional sourdoughs. At the end of a 24h fermentation cycle a pH below 3.5 can be achieved. Such specific fermentation conditions affects the selection of dominant microbiota and provides growth advantages to thermophilic and acid tolerant LAB such as *Lactobacillus panis, Lb. pontis* and *Lactobacillus amylovorus* (Stolz and Böcker, 1996). As a rule, the growth of indigenous yeast is inhibited due to the elevated fermentation temperature, and thus Type II sourdoughs are mainly used only as dough acidifiers. The leavening of the final dough is achieved by adding baker's yeasts.

Mature Type II sourdoughs are often used to produce Type III sourdoughs (Corsetti, 2013) which are typically spray– or drum–dried sourdough preparations. They are composed of LAB strains characterized by their ability to survive the drying process: facultative heterofermentative *Pediococcus pentosaceus* and *Lb. plantarum* as well as obligately heterofermentative *Lb. brevis* (Stolz and Böcker, 1996). The drying process stabilizes the sourdough and thereby increases its shelf–life. At an industrial level they are used as acidifying supplements and aroma carriers (Corsetti, 2013).

1.3.3 Initiation of fermentation process and its impact on sourdough microbiota

Dough fermentation can be initiated in various ways (De Vuyst and Neysens, 2005):

1. Relying on indigenous microbiota within the raw materials (mainly flour) used in the dough preparation – this process is termed "spontaneous fermentation"
2. Addition of "mother dough" – a part of mature sourdough from the previous fermentation cycle. This type of sourdough propagation is also known as "backslopping"
3. Addition of commercial starter culture

1.3.3.1 Spontaneous fermentation

Microflora within rye flour is composed of both bacteria and fungi and are present in amounts that vary between 10^4 – 10^6 colony forming units (CFU) g^{-1} (De Vuyst and Neysens, 2005). Bacteria are mainly mesophilic and include Gram–negative aerobic rods (*Pseudomonas, Acinetocacter* etc.), facultative anaerobes (*Enterobacteriaceae*), Gram–positive homo– and heterofermenative LAB (De Vuyst and Neysens, 2005, Minervini ert al., 2014). The fungi present are dominated by yeasts (*Candida, Pichia, Cryptococcus, Torulaspora, Saccharomyces* etc.) and moulds. The latter can originate from both the field (*Cladosporium, Fusarium, Alternaria* etc.) or from storages (*Aspergillus, Penicillium*) (Stolz, 2003; De Vuyst and Neysens, 2005).

A fresh mixture of flour and water is rich in carbohydrates and has a neutral pH (~6.2). This nutritional environment is ideal for the growth of indigenous microorganisms within flour. Studies on spontaneously fermented sourdoughs have revealed that initial fermentation is caused by bacteria that belong to the *Enterobacteriaceae* family (Stolz, 2003; De Vuyst and Neysens, 2005). These microorganisms produce lactic and acetic acids as well as ethanol and carbon dioxide.

When the pH drops below 5.0, the indigenous LAB within flour gain a growth advantage and rapidly replace gram–negative enterobacteria. Homofermentative LAB such as *Lb. delbrueckii* and *Lb. farciminis* begin to develop*,* together with facultatively heterofermentative species such as *Lb. casei* and *Lb. plantarum,* obligatory heterofermentative LAB such as *Lb.brevis* and *Lb. fermentum,* and pediococci (*Pediococcus acidilactici, Pc. pentosaceus*) (De Vuyst and Neysens, 2005). In mature spontaneous sourdoughs LAB can be found in numbers 8 – 9 log $CFUg^{-1}$ (Stolz, 2003).

The yeast species found in mature spontaneous rye sourdough are dominated by *Saccharomyces turbidans*, *Saccharomyces marchalianus*, *Torula albida*, *Kazachstania exigua (synonym Saccharomyces exiguus; anamorph Candida (Torulopsis) holmii)*, *Saturnispora saitoi* and *Saccharomyces cerevisiae*. Because *S. cerevisiae* is not typically found in the raw material, it can be assumed that its presence in spontaneous sourdoughs is mainly due to contamination from the bakery environment (Stolz, 2003; De Vuyst and Neysens, 2005).

1.3.3.2 The addition of mature sourdough

For traditional breadmaking, the use of random microflora of spontaneously started sourdoughs creates a high risk of forming an unstable end product. Instead, traditional bakeries, and many industrial bakeries, prefer to use a so called “mother dough”. The latter is a mature sourdough whose microbial population demonstrates stability in a long–term sourdough propagation cycle. It can be obtained from backslopping of spontaneously started sourdough after a number of refreshment cycles (Stolz, 2003; De Vuyst and Neysens, 2005).

Microbiological analysis of this type of fermented dough usually reveals the presence of some microorganisms typical for spontaneously fermented sourdoughs (enterobacteria, homofermentative LAB), however, the dominant microflora is generally composed of heterofermentative LAB species (De Vuyst and Neysens, 2005).

More than 60 LAB species have been isolated from different backslopped sourdoughs (Rosenquist and Hansen, 2000; De Vuyst et al., 2002; DeVuyst et al., 2014; Gänzle and Ripari, 2016). Of these, *Lb. plantarum* and *Lb. sanfranciscensis* are reported as the most frequently found (about 50% of sourdoughs) species (Gänzle and Ripari, 2016). Despite the biodiversity of sourdough related LAB, none of these species can be considered as exclusively sourdough (Gänzle and Ripari, 2016).

Yeast cells are often present in large numbers in this type of sourdough. More than 20 yeast species were detected in mature sourdoughs (De Vuyst and Neysens, 2005; De Vuyst et al., 2016). Among them, *K. exigua*, *Pichia kudriavzevii* (synonym *Issachenkia orientalis;* anamorph *Candida crusei*), *C. humilis and Pichia saitoi* were isolated from rye sourdoughs (Stolz, 2003; De Vuyst et al., 2014). Some of these yeasts are maltose–negative, such as *K. exigua*, *C. humilis* and *C. krusei* and form associations with lactobacilli in sourdough (De Vuyst and Neysens, 2005; Corsetti and Settanni, 2007). Thus, both *K. exigua* and *C. humilis* are not able to utilize maltose, and grow at excess of glucose which accumulates in the sourdough environment due to the maltose phosphorylase activity of some heterofermentative LAB such as *Lb. brevis*, *Lb. pontis* and *Lb. sanfranciscensis*. (Stolz et al, 1995).

The difference in the composition of microbial communities within spontaneously started and propagated sourdoughs also affects the quality of the baked bread. Rye breads prepared from spontaneously fermented dough have a low volume and an “empty“ taste due to an insufficient amount of technologically valuable metabolites (lactic and acetic acid, volatiles). In contrast, breads prepared from backslopped sourdough have a right consistency and classic aroma of rye bread (Stolz, 2003).

1.3.3.3 The addition of starter culture

As with any other fermented food application the interest in using starter cultures for breadmaking is rapidly growing. Despite the fact that backslopping is considered as a reliable method for the selection of sourdough adapted LAB strains, the formation of a stable microbial community by long–term propagation is time consuming and the end result always depends on both the indigenous microbial consortia within the raw materials and the applied fermentation conditions (De Vuyst and Neysens, 2005; Van der Muelen et al., 2007; Di Cagno et al., 2014; Minervini et al., 2015). Under unstable propagation conditions, microbiota within the mother sourdough could significantly vary in both the number of microorganisms and their proportions (Ottogalii et al., 1996). In order

to ensure that the quality of the end product remains stable, some industrial bakeries prefer to use commercial starter cultures to initiate the fermentation process (De Vuyst and Neysens, 2005).

Starter cultures have been defined as pure or mixed culture preparations of live microorganisms or their resting forms whose metabolic activity is well–defined and whose addition to the raw material results in the production of fermented foods of desired quality in a shorter period of time (Vogel et al., 2011). Hence, starter cultures for baking applications should be able to produce properly acidified sourdough for further use as both a flavor carrier, texture–improving, and functional dough ingredient (De vuyst et al., 2009).

Starter cultures used in baking industry can be classified into two groups. The first group are the so called "undefined" cultures such as the San Francisco sour for wheat bread production (Kline and Sugihara, 1971) or the Böcker–Reinzucht–Sauer used as sourdough starter for rye bread production (Böcker, Vogel and Hammes, 1990). These starters of undefined microbial consortia are subjected to continious changes in their specific composition due to the phage attacks, mutations, and intraspecific competition (Vogel et al., 2011). The second group are termed "defined cultures" – single– or multi–strain starter, whose carefully selected specific composition allowes a higher level of control over the dough fermentation process (Vogel et al., 2011). In recent years, both single– and multiple strain freeze–dried starter cultures have been developed. Starters that contain pure cultures of *Lb. sanfranciscensis, Lb. paralimentarius, Lb. pontis, Lb. amylovorus*, *Lb. delbrueckii*, *Lb. fermentum, Lb. reuteri, Lb. plantarum,* and *Lb. brevis* as well as a wide variety of mixed–cultures are available for the use in baking industry (Gänzle, Vermuelen and Vogel, 2007, Weckx et al, 2010).

There are three main criteria used to select starters (Coda et al., 2014):

1) Technological (acidification rate; osmotic sensitivity; the growth at selected temperature; synthesis of antibacterial and antifungal compounds etc. (Coda et al., 2011; Coda et al., 2014)
2) Sensory (synthesis of aroma compounds or their precursors; heterofermentative metabolism; release of free amino acids etc.(Gobbetti et al., 2005; Petell, Onno and Prost, 2017)
3) Nutritional (degradation of anti–nutritional factors; synthesis of polysaccharides etc. (Coda et al., 2014)

In general, selection of starter bacteria for industrial needs often relies only on a small number of specific technological parameters such as rapid acidification. In addition, allochthonous starter cultures that originate from non–cereal matrixes are commonly used (Leroy and De vuyst, 2004; Coda et al., 2014). Commercial allochthonous starters have several limitations. In general, their selection does not take into account features other than fast acidification. In addition, they possess a low metabolic flexibility and poor adaptation to the main

nutritional and functional properties of the current matrix. As a result, allochthonous starter strains are often outcompeted in long–term propagation process by autochthonous microorganisms which often results in fermentation failure and an unstable final quality of the baked products (De Vuyst et al., 2009; Coda et al., 2014).

One challenge in fermenting rye dough is balancing the ability to perform rapid acidification while forming a suitable sensory profile, producing shelf–life extending compounds, and ensuring the robustness of the starter in a sourdough cycle. Recent studies show that selection of starter bacteria based on specific functional properties should be more focused on autochthonous LAB strains and should be combined with studies of both microbial growth kinetics and intraspecific associations with other autochthonous microorganisms in the matrix of interest (Minervini et al., 2010; Coda et al., 2014).

1.3.4 Factors that influence the stability of traditional sourdoughs

The microbial communities within sourdoughs depend on both endogenous and exogenous factors (Hammes and Gänzle, 1998; De Vuyst and Neysens, 2005; Vogelmann and Hertel, 2010; Di Cagno et al., 2014). The main endogenous factors that affect microbial communities within sourdoughs are the chemical and microbiological compositions of the raw materials, while the main exogenous factors include technological parameters, such as temperature, dough yield, and aeration (De Vuyst and Neysens, 2005; Huys, Daniel and De Vuyst, 2013). The impact of these parameters and their combinations during backslopping results in both the selection of sourdough–specific LAB and yeasts and aids in preventing the growth of other microorganisms that originate from non–sterile raw materials or the bakery environment (De Vuyst and Neysens, 2005).

1.3.4.1 Endogenous factors

The flour used to propagate traditional sourdoughs may influence the stability of microbial consortia in various ways. Despite the fact that traditional sourdoughs are usually propagated using the same type of flour, the nutritional composition of the latter can vary depending on the harvest conditions. With the exception of very robust microorganisms, even small variations in substrate quality may affect the sourdough microbiota, because their ability to adapt to specific substrates is highly strain–specific (Vogelmann et al., 2009; Minervini et al., 2012; Minervini et al., 2014). In addition, flours used in backslopping processes are not sterile, and are thus a source of contaminating microorganisms. Those microorganisms, in turn, could outcompete some of the sourdough strains and even become dominant (De Vuyst et al., 2009).

Apart from the raw materials used in sourdough processes, “house” microbiota is another important source of microbial contaminants. It has been reported that microorganisms that colonize the bakery environment and

equipment can influence both the stability of and microbial composition of traditional sourdoughs (Scheirlink et al., 2009; Minervini et al., 2015).

1.3.4.2 Exogenous factors

Besides raw materials and the bakery environment, which are responsible for the metabolic activity and diversity of microbial contaminants, specific sourdough fermentation parameters such as dough yield (DY), fermentation temperature, fermentation time, inoculum size, redox potential, and aeration play a crucial role in stabilizing traditional sourdoughs (Minervini et al., 2014; De Vuyst et al., 2014).

DY can be determined as the ratio between dough weight and flour weight multiplied by 100 $\left(\mathrm{DY} = \frac{\text{flour weight} + \text{water weight}}{\text{flour weight}} \times 100\right)$. Because water, together with flour, are the main ingredients of dough, DY describes the amount of water used in the dough (Lund, Hansen and Lewis, 1989). The DY of traditional sourdoughs lies in the range of 150 – 225, which translates to a dough rheology anywhere between firm and semi–liquid (Kulp, 2003). The DY has a direct influence on the water activity and acidity of the sourdough. The sourdoughs with low DY provide microorganisms with a higher concentration of carbohydrates and a lower water activity compared with high DY sourdoughs. Because LAB are generally less tolerant to low water activity than yeasts, sourdoughs with low DY are easily colonized by the latter, however, this also creates a more selective environment for LAB. (Corsetti et al., 2007; Minervini et al., 2012b; Vrancken et al., 2011; Zotta et al., 2009). More liquid sourdoughs are characterized by faster and stronger relative acidification, despite the fact that the production of acetic acid is generally lower in sourdoughs with high DY (Decock and Cappelle, 2005; Banu et al., 2011).

Traditional sourdough propagation processes runs under semi–anaerobic conditions. However, oxygen can get into sourdough at the beginning of a new fermentation cycle, when a portion of the mature dough is mixed with a fresh portion of flour and water (Mihhalevski et al., 2011; Viiard et al., 2012). The level of oxygen influences the redox potential of the dough (Minervini et al., 2014). As the result, LAB that have an ability to use oxygen as external electron acceptors may gain an ecological advantage in aerated doughs (De Vuyst and Nyensens, 2005; Mihhalevski et al., 2011). Sourdough yeasts are usually less affected by the presence or absence of oxygen. However, some species such as *P. kudriavzevii* can only grow under aerobic conditions and thus prefer sourdoughs with high DY where aeration occurs both due to the stirring and pumping (Vogelmann and Hertel, 2010).

The initial pH of the dough is another important factor that affects the stability and dynamic shifts in the microbial community within traditional sourdoughs (Minervini et al., 2014; De Vuyst et al., 2014). The initial pH of sourdough is determined by the share of mature sourdough (“mother dough”) used for backslopping. In traditional sourdoughs the percentage of ripe

sourdough used for refreshment lies in range of 10 to 40% of the total dough weight (Brandt, Hammes and Gänzle, 2004). The higher the share of the "mother dough", the lower the initial pH of the dough. Thus, a high ratio of "mother dough" inhibits the growth of sourdough lactobacilli, yet does not affect the growth of acid–tolerant sourdough yeasts (Gänzle, Ehrmann and Hammes, 1998; Brandt, Hammes and Gänzle, 2004).

Besides DY, pH, and redox potential, fermentation temperature is considered as important parameters that determine the stability and metabolite kinetics of sourdough (Minervini et al., 2014; De Vuyst et al; 2014). Seasonal or even daily temperature changes may affect the development of microbial communities in traditional sourdough (Vera et al., 2012) and, consequently, the ratio of lactic to acetic acid. A shift towards lactic acid production occurs at higher fermentation temperatures (De Vuyst et al; 2014) because higher fermentation temperatures favor the growth of homofermentative LAB. In contrast, lower fermentation temperatures positively influence the growth of yeasts and heterofermentative LAB species and favor the production of acetic acid, ethanol, and flavour formation (Gänzle, Ehrmann and Hammes, 1998; Moroni, Arendt and Dal Bello, 2011; Vogelman and Hertel, 2011). Finally, high fermentation temperature may negatively affect the mutualistic association between maltose–positive LAB and maltose–negative yeasts because the latter are not able to grow at temperatures above 35°C (Gänzle, Ehrmann and Hammes, 1998; Brandt, Hammes and Gänzle, 2004; Vogelman and Hertel, 2011).

1.4 Microbiological spoilage of bread

As with any other food product with a high water activity, breads are subject to microbiological spoilage caused by bacteria and fungi (Saranraj and Geetha, 2012). The most common bacterial bread disease, called "rope", is caused mainly by the spore forming bacteria *Bacillus subtilis,* however, other species within the genus *Bacillus,* including *Bacillus cereus*, *Bacillus licheniformis,* and *Bacillus megaterium* are also capable of causing "rope". Spores of rope–inducing bacilli are widely found in raw ingredients such as flour, sugar, and yeast biomass and are resistant to heat treatment, which makes them a common problem for bakeries (Smith, 1993). Moreover, spores of these *Bacillus* species can germinate and grow under both aerobic and anaerobic packaging conditions (Smith et al., 2004). One of the main factors that limit the growth of roping bacilli is low pH (Pepe et al., 2003). Traditional sourdough rye breads have much lower pH (3.5 – 4.8) compared to wheat breads, which makes them resistant to bacterial spoilage. Thus, fungal spoilage is the main factor that limits the shelf–life of rye breads (Legan, 1993).

Both yeasts and molds are associated with the fungal deterioration of bread. There are two types of yeast spoilage – fermentative spoilage and visible growth on the surface of the bread. The latter results in the development of white spots on the surface of the infected product – a phenomenon known as "chalk" bread

disease. The two main yeast species associated with chalk bread defect are *Hyphopichia burtonii*, also known as "chalk–mould" and *Endomyces fibuliger* (Deschuyffeleer et al., 2011). However, in a study conducted by Lund et al. (1996), *Pichia anomala* was reported as one of the dominant yeast species isolated from spoiled rye breads.

Together with yeasts, moulds create a serious and costly problem for the baking industry. According to Malkki and Rauha (2000) up to 5% of the total yearly bread production worldwide may be lost due to mould spoilage. Mould spores are killed by heat treatment during the baking process which implies that post treatment contamination takes place through the air and from equipment during cooling, slicing, and packaging (Legan, 1993). Although rye breads can be contaminated by a range of different moulds, it seems that *Penicillium* and *Aspergillus* species are the most predominant (Lund et al., 1996). *Penicillium verrucosum, Aspergillus ochraceus, Aspergillus bombycis* and many other representatives of these two genera are well known mycotoxin producers, including aflatoxins B1, B2, G1, and G2, and ochratoxin A (Varga, Frisvad and Samson, 2011; Schmidt, 2003,). Considering that Dich et al. (1979) found an aflatoxin producing *Aspergillus flavus* in spoiled rye bread and an ochratoxigenic *A. ochraceus* was isolated from moldy bread in Italy (Visconti and Bottalico, 1983), it is clear that besides the economic losses, mould spoilage of bread could also lead to public health problems.

1.5 Sourdough LAB as biopreservative agents for baking industry

As discussed above, the fungal spoilage of rye bread is a big problem within the baking industry. In Western Europe alone, it is estimated that the baking industry loses more than 200 million euros annually due to fungal spoilage (Pawlowska et al., 2012). A broad range of chemicals is used in the battle against spoiling fungi (Brul and Coote, 1999; Davidson, 1999). However, legislative restrictions regarding the use of chemicals for food production, together with a rapidly growing trend in "green", additives–free food products, has resulted in the development of a new trend known as "biopreservation".

Biopreservation can be defined as the extension of the shelf–life of food by the use of microorganisms, typically through the production of several natural antimicrobial agents (Leroy and De Vuyst, 2004; Pawlowska et al., 2012). LAB can be considered very promising biopreservative microorganisms because they are generally recognized as save (GRAS) and are used as starter cultures for the manufacture of a wide variety of foods (Leroy and De Vuyst, 2004). LAB produce a range of antimicrobial compounds such as organic acids, hydrogen peroxide, proteinaceous compounds, and reuterin (multi–compound consisted from 3–hydroxypropionaldehyde, its hydrate and its dimer) (Corsetti and Settannii, 2007; Axel, Zannini and Arendt 2016). Some of these antimicrobial compounds are resistant to the baking process and can control the growth of spoilage fungi.

It has been reported that, in addition to their influence on dough rheology, loaf volume, crumb structure and bread aroma, dough fermentation, in particularly sourdough LAB, also play a crucial role in the extension of the shelf–life of bread (Gerez et al., 2009; Lavermicocca et al., 2000; Dal Bello et al., 2007; Ryan et al.,2011). Dal Bello et al. (2007) revealed that *Lb. plantarum* FST 1.7 has the ability to inhibit bread spoilers that belonged to the genus *Fusarium.* The Main antifungal compounds identified in that study were lactic and phenyllactic acids, and two cyclic dipeptides. Another strain of *Lb. plantarum* was examined in a study conducted by Lavermicocca et al. in 2000. As a result, the tested strain demonstrated the ability to inhibit *Aspergillus niger* growth in both sourdough and prepared breads. They identified acetic, lactic and phenyllactic as antifungal compounds. Moreover, in some cases, the antifungal activity of tested LAB strains even exceeded the efficiency of some chemical preservatives (Lavermicocca et al., 2000; Pawlowska et al., 2012).

Research exploring the use of antifungal compounds produced by sourdough LAB is still quite novel. Only a limited number of inhibiting compounds have been described, and, in some cases their mechanisms of action remain unclear (Brul and Coote, 1999; Schnürer and Magnusson, 2005; Dalie, Descamps and Richard–Forget, 2010). However, the potential use of LAB with antifungal properties seems to be very attractive for traditional breadmakers both for legislatory and marketing reasons and can help to find a balance between consumer demands and economic losses caused by fungal contamination.

3. AIMS OF THIS DISSERTATION

The aim of this work was to select functional starter LAB strains for Type I sourdough processes. We obtained these strains from mature spontaneously started Type I rye sourdough after two months of controlled propagation using critical technological parameters that determine the stability of industrial rye sourdoughs as selective factor. We then characterized the dominant lactic acid bacteria in order to evaluate their potential as robust functional starter cultures for Type I sourdoughs. Specifically, we studied the following:

I. Factors that affect the performance of dough fermentation in bakeries that use traditional sourdough processes (Publication I)

II. Characteristics of the dominant strains isolated from spontaneously started Type I rye sourdough after a long–term controlled fermentation under selective pressure. Here we assessed the potential of selected strains for application in Type I sourdough fermentation processes and their ability to prolong the mould–free shelf life of rye bread (Publications II and III)

III. The diversity of contaminating fungi isolated from rye breads purchased from various Estonian bakeries. The aim here was to create a collection of mould species for further antifungal studies (Publication IV)

4. MATERIALS AND METHODS

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

4.1 Sourdough samples

4.1.1 Industrial sourdough samples

Industrial sourdough samples were originated from four Estonian bakeries referred to as A_{bakery}, B_{bakery}, C_{bakery}, and D_{bakery}. All sourdoughs were prepared from flour of the same origin (rye flour type 1370, extraction rate 85%, Tartu Mill AS, Estonia).

Table 1. Industrial sourdough samples collected from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) and their propagation conditions. In C_{bakery} and D_{bakery} the fermentation was carried out at room temperature (RT). "Months" indicate time passed from the beginning of a new sourdough cycle (A_{bakery}, B_{bakery}, and C_{bakery}) or from the collection of the first sourdough sample (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)
Dough yield	250	400	~ 200	~ 200
Analyzed samples	A0 – freeze – dried sourdough	Bs – 3 years propagated sourdough	C0 – freeze – dried commercial starter	D1 – approx. 30 years propagated sourdough (19°C)
	A1 – 1.2 months	B0 – fresh sourdough starter	C1 – 12 months	D2 – 3 months (30°C)
	A2 – 3.5 months	B1 – 0.25 months	C2 – 21 months	D3 – 5 months (28°C)
	A3 – 4.5 months	B2 – 1 month	C3 – 28 months	D4 – 19 months (23°C)
	A4 – 8.5 months	B3 – 2.5 months		
		B4 – 6 months		

Based on the propagation technology employed at each bakery (Table 1) A_{bakery} and B_{bakery} sourdoughs can be referred to Type II, whereas C_{bakery} and D_{bakery} sourdoughs are classified as Type I. In A_{bakery} and B_{bakery}, sourdough propagation was carried out under a controlled temperature, while C_{bakery} and D_{bakery} propagated sourdoughs at ambient temperature, which fluctuated seasonally. A number of relevant sourdough propagation parameters are described in Publication I: fermentation temperature and time, inoculum size, dough yield, starter used to initiate fermentation, and sampling schedule.

4.1.2 Laboratory sourdough samples

4.1.2.1 Spontaneously started sourdough

Sourdough fermentation was initiated by mixing rye flour and sterile 0.5% NaCl solution in proportion 1:1. Rye flour (Type 1370, Tartu Mill, Estonia) from a single 50 kg bag was used for all experiments. After mixing, three sourdough batches were fermented at 20°C and another three at 30°C. After 24 hours of fermentation, each sourdough was renewed at an inoculation rate of 1:10. 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 (Publication II).

4.1.2.2 Sourdoughs inoculated by single–culture starter bacteria

Starter LAB (*Lb. plantarum* M30I–1, *Lb. brevis* M30I–2, *Lb. paralimentarius* M30I–3 and *Lactobacillus crustorum* M30I–9) were cultivated overnight at 30°C in Sourdough bacteria (SDB) broth (maltose 20 gl^{-1}, Tween 80 0.3 gl^{-1}, trypticase 5 gl^{-1}, pH5.6). We prepared four dough batches using 10 KGy irradiated rye flour and sterile 0.5% NaCl solution (1:1). Each batch was inoculated with a single culture of starter LAB to achieve a final concentration of 10^6 $CFUg^{-1}$ bacterial cells in the dough. After mixing, each dough was divided in 2 portions and incubated for 12h. One portion was incubated at 20°C and another at 30°C (Publication III).

4.1.2.3 Sourdoughs inoculated by mixed–culture starter

Starter LAB were cultivated as described in the previous paragraph. Maltose negative yeast *Candida humilis* SR1 was cultivated overnight at 30°C in Yeast Extract Peptone Dextrose (YPD) broth (dextrose 20 gl^{-1}; peptone 20 gl^{-1}; yeast extract 10 gl^{-1}). Doughs prepared as described above were inoculated with a single culture of selected starter LAB together with *C. humilis* SR1 to achieve a final concentration of 10^6 CFU g^{-1} bacterial cells and 10^4 CFU g^{-1} yeast cells in the dough. After mixing, each sourdough was divided into 2 portions and incubated for 12h. One portion was incubated at 20°C and another at 30°C. After 12 hours of fermentation, the sourdoughs were refreshed 1:10. In total, each experimental sourdough went through fourteen refreshment cycles(Publication III).

4.2 Analysis of sourdough samples

4.2.1 Chemical analysis

I. For analysis of pH and total titratable acidity (TTA) the sourdough was homogenized with distilled water using a Polytron PT2100 homogenizer (Kinematica AG, Switzerland). TTA and pH were measured using a DL22 Food and Beverage Analyzer (Mettler–Toledo LLC., USA) (Publications I, II and III).

II. Concentrations of lactic and acetic acids in mature sourdoughs were measured using High Performance Liquid Chromatography (HPLC). A Separations Module Waters 2695 Alliance HPLC was used together with a Refractive Index Detector 2414 (both from Waters Corporation, USA) and Organic Acid Analysis column (Aminex HPX–87H ion exclusion column, 300 mm X 7.8 mm (Bio–Rad Laboratories Inc., USA)). A sulfuric acid solution (2.2 ml per 1 l MilliQ water) was used as a solvent with flow rate 0.6 ml min^{-1}, at a temperature of 35°C (Publication III).

III. The extraction of volatile compounds from the dough was carried out using solid–phase microextraction (SPME). Analysis of the volatile compounds in the sourdoughs was performed using a Micromass GCT Premier gas chromatograph system (Waters, Milford, MA, USA) coupled with a PAL autosampler (CTC Analytics AG, Lake Elmo, MN, USA). After the SPME procedure, the volatile compounds were desorbed in splitless mode into a GC injection port equipped with a 0.75 mm internal diameter liner at 250°C for 10 minutes. A DB5–MS column (30 m length × 0.25 mm i.d. × 1.0 µm film thickness; J&W Scientific, Folsom, CA, USA) was used with helium as a carrier gas at a flow rate of 1.0 ml min^{-1}. The identification and quantification of volatile compounds was carried out using MassLynx software (Waters, Milford, MA, USA) (Publication III).

4.2.2 Microbiological analysis

I. Bacterial counts were determined by planting on SDB and de Man, Rogosa and Sharpe (MRS) agar (Lab M Ltd, UK) agar containing 100 μgl^{-1} cycloheximide (Sigma–Aldrich, USA). CFUs were counted from agar plates with suitable dilutions (Publications I and II and III).

II. Yeast counts were measured by plating on YPD agar plates containing 100 μgl^{-1} chloramphenicol (Sigma–Aldrich, USA). Petri dishes were incubated overnight at 30°C under aerobic conditions. CFUs were counted from agar plates using suitable dilutions (Publication II and III).

4.2.3 Total DNA extraction and cultural–independent analysis of sourdough microbiota

I. Extraction of whole DNA was performed using a GenElute Bacterial Genomic DNA Kit (Sigma–Aldrich., USA), according to the manufacturer's protocol (Publications I and II).

II. To conduct a Denaturing Gradient Gel Electrophoresis (DGGE) analysis of the yeast community we amplified the 28S rRNA genes using primers U1GC and U2. To conduct pyrosequencing analysis V1–V2 hypervariable regions of 16S rRNA were amplified using universal primers 8F and 357R (Publications I and II).

III. Denaturing Gradient Gel Electrophoresis (DGGE) was carried out using the INGENYphorU system (Ingeny International Bv., Netherlands). All clearly visible bands were cut from the gel, purified, and reamplified using primers or U1 and U2 (Publications I).

IV. For Agarose gel electrophoresis (AGE) analysis of yeast community amplification of Internal Transcribed Spacer (ITS) region was made using primers ITS1 and ITS4. Obtained amplicons were visualized on agarose gel, sliced out, purified and reamplified using primers ITS1 and ITS4 (unpublished data).

V. Sanger sequencing of selected amplicons was conducted 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) (Publications I and II).

VI. Pyrosequencing of Bar–coded 16S rRNA Gene Amplicons was made 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. Operational Taxonomic Units (OTUs) were defined using the average neighbor clustering algorithm within MOTHUR with a 97% similarity threshold. The closest match at a species level was found for each OUT using the BLASTn algorithm together with the GenBank database (National Center for Biotechnology Information, USA) with a similarity of 97% and a coverage of 90% (Publications I and II)..

4.3 Bread samples

The ten sliced sourdough rye breads studied in Publication IV were produced by five different Estonian bakeries, designated A, B, C, D and E. Loaves were made without any preservatives and packed into plastic bags.

4.4 Identification of microbial isolates

I. Bacterial DNA was extracted from isolated colonies using Whatman indicating FTA MiniCards (GE Healthcare Ltd., UK) and the method provided by the manufacturer (Publications I and II). Fungal DNA was extracted using phenol/chloroform/isoamyl alcoholic extraction (Publication IV).

II. PCR–fingerprinting analysis of bacterial and fungal isolates was conducted using the primers $(GTG)_5$ (Publications I and II) and M13 (Publication IV), respectively. PCR–fingerprints were visualized on agarose gel.

III. To identify bacterial isolates, 16S rRNA gene fragments were amplified using the universal primers 27f–YM and 16R1522 (Publications I and II). Fungi were identified by amplifying the D1/D2 variable domains of the 28S rRNA gene, Internal transcribed spacer (ITS) region, and the β–tubulin gene with the primer pairs NL1/NL4, ITS1/ITS4, and Bt2a/Bt2b, respectively (Publications I, II and IV).

IV. Sanger sequencing of the amplicons was performed at 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) (Publications I, II and IV).

4.5 Characterization of dominant LAB isolated from spontaneously fermented sourdough

LAB used in the study: *Lb. plantarum* M30I–1, *Lb. brevis* M30I–2, *Lb. paralimentarius* M30I–3 and *Lb. crustorum* M30I–9 were isolated from spontaneously started laboratory rye sourdough (Publication II).

I. We studied the carbohydrate fermentation profiles of selected LAB strains using API 50 CH identification kit according to the method provided by the manufacturer (bioMe´rieux, France) (Publication II).

II. The growth of single–culture bacteria in dough at different temperatures was characterized using an isothermal microcalorimeter TAM III (Publication III)

III. The ability of selected LAB to suppress the growth of reference microorganisms was tested in both an agar well diffusion assay and dual culture overlay assay. The reference microorganisms used for this study: *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Yersinia enterocolitica* ATCC 27729, *Escherichia coli* ATCC 25922 and *Aspergillus brasiliensis* ATCC 16404 were kindly provided by the Laboratory of Food and Environmental

Investigations (Institute of Food Safety, Animal Health and Environment – "BIOR", Latvia) (Publication III).

4.6 *In situ* study of the antifungal activity of *Lactobacillus brevis* M30I–2 against bread spoiling fungi

Reference mould strains *A. niger* B213, *Aspergillus tubingensis* B123, *Aspergillus chevalieri* D121, *Penicillium corylophilum* A111 and *Penicillium citrinum* D221 were isolated from mouldy rye bread (Publication IV).

4.6.1 Bread making

Sourdough for bread making was prepared as described in Section 4.1.2.2. Rye dough was mixed according to the following recipe: sourdough – 33%, sterile water – 28%, sterile flour – 39%. These doughs were fermented for 110 minutes at 30°C, put into baking forms and proofed at 30°C for an additional 45 minutes. Baking was carried out in a Self Cooking Centre (Metos Systems Rational, Weikersheim, Germany) as follows: 10 minutes at 230°C, 15 minutes at 200°C, 15 minutes at 180°C, and 10 minutes at 150°C. The baked breads were cooled to room temperature, sliced and placed in petri dishes. Both the cooling and slicing procedures were conducted under sterile conditions (Publication III).

4.6.2 *In situ* antifungal assay

Spores of reference moulds were collected from the surface of the plates by shaking them with 1.5 ml of sterile 0.5% NaCl solution. The concentration of mould spores was determined using a hemocytometer and adjusted to 10^6 spores ml^{-1}. Bread slices were spot inoculated by 5µl of spore suspension or using a piece of reference mould mycelium 5 mm in diameter and stored at 25°C for a week. Visual sings of mould germination were checked daily (Publication III).

5 RESULTS AND DISCUSSION

5.1 Impact of propagation parameters on the stability of industrial sourdoughs (Publication I)

Fermentation of the dough is a crucial step for rye bread making. Both the activity and stability of sourdough microbiota available for successful fermentation of the dough depend on multiple factors. In Publication I, we studied the effect of fermentation parameters applied at the industrial level on the stability of rye sourdoughs obtained from two large–scale (A_{bakery} and B_{bakery}) and two small–scale (C_{bakery} and D_{bakery}) Estonian bakeries.

Sourdoughs collected from both large–scale bakeries demonstrated remarkable stability in both bacterial cell density and chemical parameters (Table 2). The mean LAB counts were 8.95 log $CFUg^{-1}$ and 8.67 log $CFUg^{-1}$ for A_{bakery} and $B_{bakery,}$ respectively, while the average pH of the sourdoughs obtained from these bakeries was 3.64 and 3.56 throughout the study.

Table 2. pH, TTA and bacteria counts in industrial rye sourdoughs from four Estonian bakeries

	pH	TTA	log($CFUg^{-1}$)
	Mean ± STDEV		
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
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

Despite having similar pH values, B_{bakery} sourdough was characterized by a significantly higher TTA (Table 2). This could be the result of the sourdough propagation conditions because it has already been shown that the high DY and

increased fermentation temperature used in this bakery favor acidification (Minervini et al., 2014; De Vuyst et al., 2014).

Beside having stable chemical compositions, the sourdoughs that originate from both large–scale bakeries demonstrated very stable specific microbial populations. The LAB strains that dominated in A_{bakery} sourdough belonged to the species *Lb. panis*, *Lb. pontis,* and *Lactobacillus helveticus* (Fig. 1)*,* while the yeasts were represented by a single species – *Kazachstania telluris* (formerly *Saccharomyces telluris, Arxiozyma telluris*) (Fig. 2). This yeast species is known as a pathogen in rodents and cannot be referred to as a typical sourdough yeast (De Vuyst et al., 2016). However, this thermophilic yeast can potentially colonize a sourdough ecosystem due to its ability to utilize glucose and grow on glucose, ethanol, and lactic acid (Kurtzman et al., 2005).

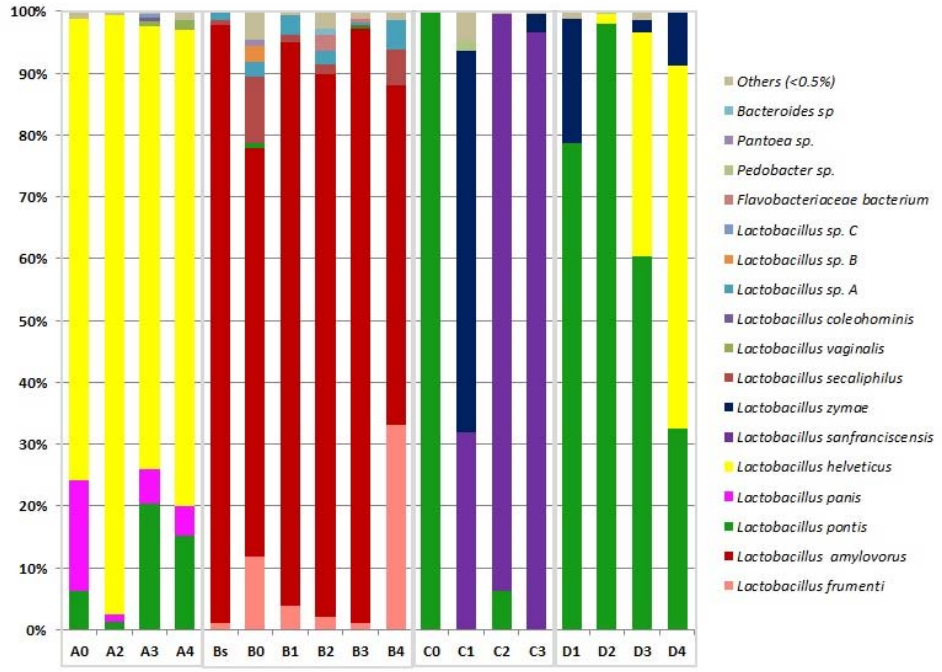

Figure 1. Diversity of bacterial communities in the industrial rye sourdoughs from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) as determined by pyrosequencing of 16S rRNA gene amplicons

The high fermentation temperature applied in B_{bakery} resulted in the selection of a stable bacterial population consisting of *Lb. amylovorus* and *Lactobacillus frumenti* (Fig. 1). Both of these thermophilic, acid–tolerant bacteria species were previously identified as prevalent in other industrial rye sourdoughs propagated at elevated fermentation temperatures, similar to that (42 – 44°C) applied in B_{bakery} (Müller et al., 2001; De Vuyst et al., 2014). No yeast species were detected in B_{bakery} sourdough, probably due to the unfavorable conditions created

by employing a high fermentation temperature (Minervini et al., 2014; De Vuyst et al., 2014).

In contrast with samples collected from large–scale bakeries where the fermentation conditions were highly controlled by industrial equipment, sourdoughs obtained from C_{bakery} and D_{bakery}, were propagated at ambient temperature and found to be much less stable. Samples collected in C_{bakery} and D_{bakery} during the winter period (samples C1, C3, D1, D4 in Table 2) were characterized by higher pH, probably due to the low ambient temperature. Changes in pH correlated with fluctuation in the LAB cell density and specific composition of the bacterial consortia and were also in direct relation with the ambient temperature within the bakery (Table 2). Thus, the sourdoughs collected in C_{bakery} during the colder period contained a microbial population dominated by *Lb. sanfranciscensis* and *Lactobacillus zymae* with the latter predominating in the coldest month (C1). During the warmer period, *Lb. zymae* was outcompeted by *Lb. pontis.*

Representatives of *L. zymae* and *L. pontis* species were also detected among the dominant population of LAB in D_{bakery}, which utilized sourdough propagation parameters that are very similar to those applied in C_{bakery}. Apart from *Lb. zymae* and *Lb. pontis* sourdough samples from D_{bakery} contained LAB from the species *L. helveticus* (Fig. 1). As with C_{bakery} sourdoughs, the relative proportion of these species in the samples depended on the ambient temperature in the bakery. In the wintertime (samples D1, D4), the growth of *Lb. zymae* was favored, while mainly *Lb. pontis* and *Lb. helveticus* were detected in samples (D2 and D3), which were collected during warmer periods (Fig. 1).

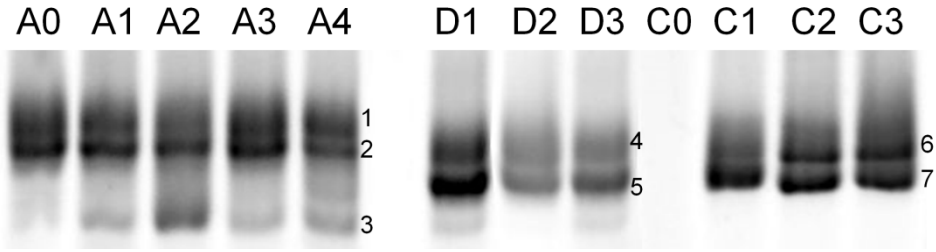

Figure 2. Identification of yeast communities within industrial rye sourdoughs from three Estonian bakeries (A_{bakery}, C_{bakery} and D_{bakery}) using DGGE analysis of 28S rRNA gene amplicons. 1, 2 – K. telluris; 3 – Cereal DNA; 4, 5, 6, 7 – C. humilis

The only yeast identified from the sourdough samples from both small–scale bakeries (C_{bakery} and D_{bakery}) belongs to *C. humilis* species (Fig. 2). In traditional sourdoughs this maltose–negative yeast species often forms a trophic relationship with maltose–positive LAB such as *Lb. sanfranciscensis* and *Lb. pontis* species (Gänzle, Ehmann and Hammes, 1998). The glucose that accumulates in the environment due to intracellular maltose phosphorylase activity of those LAB, is consumed by *C. humilis*, who is not able to utilize maltose (Hammes, Stolz and Gänzle, 1996; Martinez–Anaya, 2003). *C. humilis*,

in turn, degrades sucrose and fructose–oligosaccharides thereby liberating fructose which can be used by *Lb. sanfranciscensis* and *Lb. pontis* as an external electron acceptor, thus increasing the cell yield and production of acetic acid (Gänzle, Vermuelen and Vogel, 2007).

5.2 Selection of functional starter bacteria for traditional sourdoughs (Publications II and III)

The maintainance of stable microbial consortia in traditional sourdough may require additional investments in equipment and training for operators working in production. For artisanal and small–scale industrial bakeries that carry out Type I sourdough fermentation processes, the use of starter bacteria highly adapted to the unstable fermentation conditions could be a rational way of maintaining the quality of the rye bread produced. In this study we directed the evolution of microbial consortia to select LAB that are adapted to the parameters used to prepare traditional Type I rye sourdough. We then isolated and characterized the dominant LAB from mature long–term propagated sourdough and evaluated their potential application as bioactive, competitive, and functional starter bacteria for traditional sourdough process.

5.2.1 Selection of dominant microorganisms in spontaneously started Type I rye sourdough using fermentation temperature as a selective factor

Spontaneous rye sourdoughs were started at 20°C and 30°C in three parallel batches. Flour from the same batch was used to produce the sourdough in all experiments.

The evolution of the sourdough microbial consortia occurred more rapidly at 30°C. Thus, after the first 24 hours of fermentation at 20°C bacteria from the genera *Enterobacter*, and *Pantoea* prevailed, whereas in sourdoughs fermented at 30°C in addition to *Enterobacteria*, sourdough nonspecific LAB belonged to *Weissella*, *Lactococcus*, and *Leuconostoc* dominated (Fig. 3). The non-specific sourdough LAB produced organic acids decreasing pH and increasing the TTA of the dough (data not shown). However, pH drop below 4.0 causes inhibiting effect on sourdough non–specific LAB, favoring the growth of acid–tolerant LAB species (Minervini et al., 2014; De Vuyst et al., 2014).

At the end of the first week of experiments in doughs fermented at 30°C, non-specific sourdough LAB were partly replaced by sourdough–specific species (*Lb. plantarum* and *Lb. brevis*). In contrast *Weissella*, *Lactococci,* and *Pediococci* still prevailed in sourdoughs fermented at 20°C (Fig. 3), which were characterized by lower acidity (data not shown).

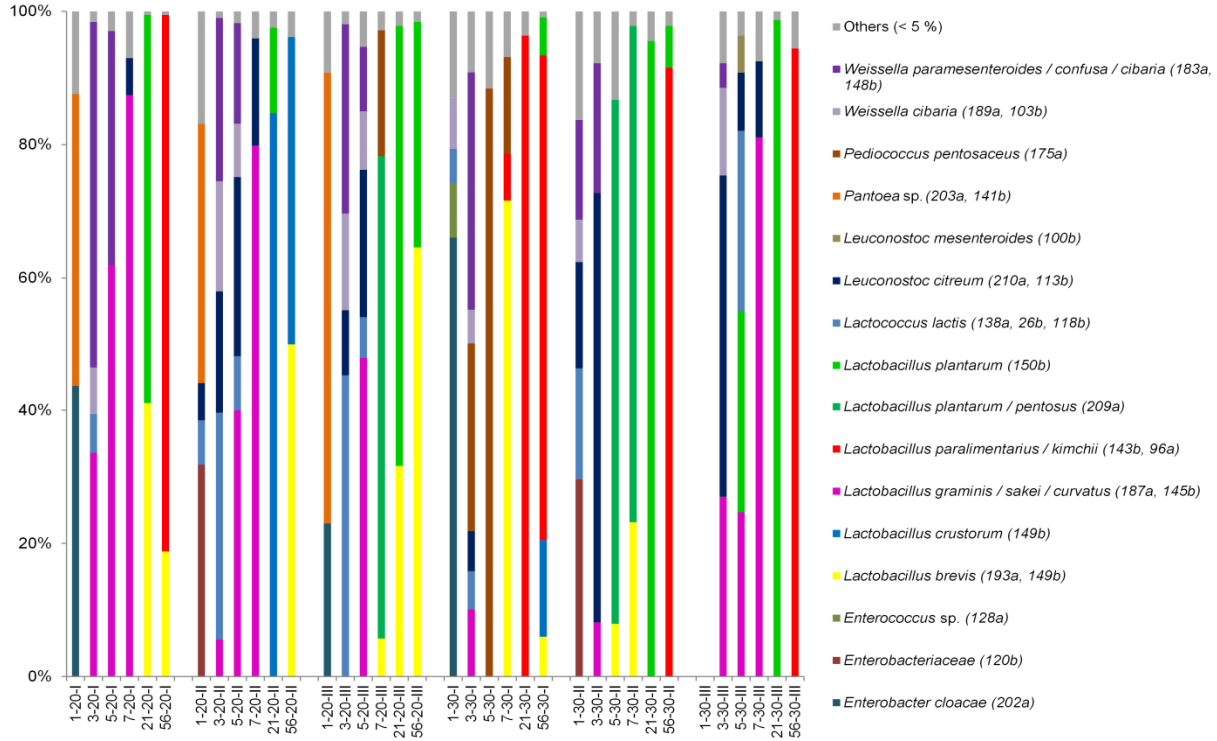

Figure 3. Pyrosequencing of 16S rRNA gene amplicons from spontaneous rye sourdoughs propagated for 56 days. Three sourdoughs were fermented at 20°C (20–I, 20–II, 20–III) and three sourdoughs fermented at 30°C (30–I, 30–II, 30–III). Sourdoughs were sampled at Day 1, 3, 5, 7, 21, and 56. The relative abundance 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". (a) and (b) stand for two different sequencing runs.

During the next two weeks of daily backslopping, the diversity of the six bacterial communities continued to decrease. Starting from the 21st cycle to the 35th cycle, the bacterial compositions within the sourdoughs were stable and comparable between batches.

Although temperature is an important parameter that determines metabolite kinetics and the establishment of microbial consortia during sourdough fermentation (Meroth et al., 2003; Vrancken et al., 2011; Minervini et al., 2014; De Vuyst et al., 2014), in this study, fermentation temperature mostly affected the ratios between the limited number of dominant LAB species in the community rather than selecting for different species. Thus, after 56 propagation cycles, facultatively heterofermentative *Lb. paralimentarius* was found to dominate in sourdoughs fermented at 30°C with different combinations of *Lb. plantarum, Lb. brevis,* and *Lb. crustorum* found at sub–dominant levels (>15%). In sourdoughs fermented at 20°C obligately heterofermentative *Lb. brevis* in combination with facultatively heterofermentative *Lb. plantarum, Lb. paralimentarius,* and *Lb. crustorum* prevailed.

In contrast with the bacterial consortia, the dynamics of the yeast population during the earlier stages of propagation was not affected by the fermentation temperature applied. The yeast community in all studied sourdoughs was generally stable during the first three weeks of daily propagation and consisted from a single yeast species – *Kazachstania unispora* (formerly *Saccharomyces unisporus*) (Fig. 4). A drastic increase in the number of yeast species appeared near the end of the first month of backslopping (day 28, Fig. 4) when we began to find yeast from the species *S.cerevisiae,* and *Candida glabrata* in sourdoughs fermented at both temperatures (Fig. 4).

During the next 28 cycles of propagation, the yeast species *S. cerevisiae* and *C. glabrata* disappeared from the sourdough batches fermented at 20°C. By the end of the experiment *K. unispora* was found to be the only yeast species in all sourdoughs fermented at the lower temperature. In contrast, sourdoughs fermented at 30°C yeast from the species *C. krusei* appeared at the 35th fermentation cycle. After the 56th backslopping cycle, the yeast populations within the sourdoughs propagated at 30°C consisted of *K. unispora* along or in combination with *S. cerevisiae, C. krusei*, and *C. glabrata* (Fig. 4).

It has been shown that the stabilization of LAB consortia in spontaneously started sourdoughs occurs in a three–stage evolution process over the course of five to ten days (De Vuyst and Neysens, 2005; Weckx et al., 2010; Ercolini et al., 2013; Moroni, Arendt and Dal Bello, 2011). During this time, the acidity drops and stabilizes together with the LAB count to a level common for mature sourdough. However, in our study further succession of species occurred even after the fifth week of propagation (Figure 3).

It is known that yeasts and LAB often interact with each other in sourdough ecosystems (Minervini et al., 2014; De Vuyst et al., 2014). Being maltose–negative, *K. unispora, C. krusei,* and *C. glabrata* can enhance the growth of maltose positive LAB, such as *Lb. brevis* (Ravyts and De Vuyst, 2011; De Vuyst

et al., 2016). Hence, the appearance of the new yeast species in some of the batches we tested may be the reason for the observed instability.

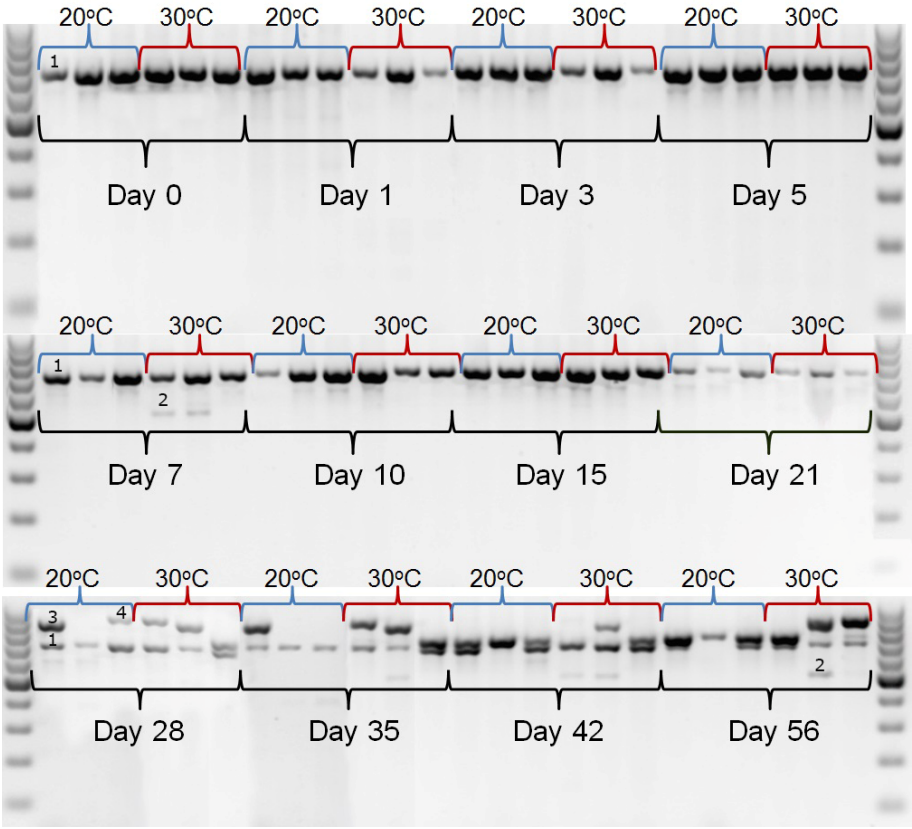

Figure 4. Identification of yeast communities within spontaneously started laboratory rye sourdoughs using AGE analysis of ITS1/4 amplicons. 1– K. unispora; 2 – C. krusei; 3 – S. cerevisiae; 4 – C. glabrata.

5.2.2 Isolation of dominant LAB strains and characterization of their metabolic profiles

In total, 120 colonies of LAB were isolated from spontaneously–started rye sourdoughs after 56 days of laboratory–scale propagation at two different temperatures. The isolates were then clustered into four REP groups based on Rep–PCR fingerprinting (data not shown). Representatives from each group were identified using 16S rRNA partial gene sequencing and were found to be *Lb. paralimentarius* (*Lb. paralimentarius* M30I–3) and *Lb. crustorum* (*Lb. crustorum* M30I–9), *Lb. plantarum* (*Lb. plantarum* M30I–1) and *Lb. brevis* (*Lb. brevis* M30I–2). The two latter species are considered as the two most frequently

isolated LAB from sourdoughs (Corsetti and Settanni, 2007; De Vuyst et al., 2014).

We went on to characterize the metabolic profiles of the four dominant LAB species. The strain *Lb. plantarum* M30I–1 was able to ferment the largest range of carbohydrates (Table 3). Such a diverse carbohydrate metabolism can explain the predominance of *Lb. plantarum* species in cereal fermentations (Minervini et al., 2010; Manini et al., 2016). Together with *Lb. paralimentarius,* M30I–3, *Lb. plantarum* M30I–1 was able to ferment all four major rye flour carbohydrates (glucose, fructose, maltose, sucrose), while *Lb. brevis* M30I–2 and *Lb. crustorum* M30I–9 were sucrose–negative (Table 3).

Table 3. Carbohydrate fermentation profiles of Lb. plantarum M30I–1, Lb.brevis M30I–2, Lb. paralimentarius M30I–3 and Lb. crustorum M30I–9

	Lb. plantarum M30I–1	*Lb. brevis* M30I–2	*Lb. paralimentarius* M30I–3	*Lb. crustorum* M30I–9
L – arabinose	+	+	+	–
D – ribose	+	+	+	–
D – xylose	+	+	+	–
D – galactose	+	+	–	+
D – glucose	+	+	+	+
D – fructose	+	+	+	+
D – mannose	+	–	+	+
D – celiobinose	+	–	+	+
D – maltose	+	+	+	+
D – melibiose	+	+	–	–
D – saccharose	+	–	+	–
D – trehalose	+	–	+	+
Inulin	+	–	–	–
D – melezitose	+	–	+	–
D – raffinose	+	–	–	–
D – mannitol	+	–	–	–
D – sorbitol	+	–	–	–
Esculin ferric citrate	+	–	+	–

5.2.3 The growth of selected LAB in Type I sourdough at 20°C and 30°C

Because fermentation temperature is one of the key factors that affects the growth of microorganisms in sourdough cycles (Minervini et al., 2014; De Vuyst et al., 2014; Vrancken et al., 2011; Neysens and De Vuyst 2005; Brandt, Hammes and Gänzle, 2004), bakeries that apply ambient temperatures for sourdough fermentation require starter cultures with an ability to grow and acidify the dough over a wide range of temperatures.

The fermentation temperatures used in our trials resulted in significantly different metabolic performance of strains we tested. When studied doughs were fermented at 30°C, all LAB strains demonstrated rapid growth with a shorter exponential growth phases and higher maximum heat flows compared with doughs fermented at 20°C (Table 4, Fig. 5).

Table 4. Growth characteristics of Lb. plantarum M30I–1(LP), Lb. brevis M30I–2 (LB), Lb. paralimentarius M30I–3 (LPR) and Lb. crustorum M30I–9 (LC) during cultivation at 20 and 30°C. μ_{max} – maximum specific growth rate, P_{max} – maximum heat flow, log(CFUg^{-1}) – bacteria counts.

Temperature	Starter	μ_{max}, h^{-1}	P_{max}, µWg-1	log(CFUg^{-1})
		Mean ± STDEV		
20ºC	LP	0.127±0.01	NR*	8.73±0.41
	LB	0.175±0.01	239.5±0.01	9.14±0.11
	LPR	0.095±0.00	NR	8.45±0.02
	LC	0.121±0.03	NR	8.64±0.00
30ºC	LP	0.472±0.02	268.6±0.01	9.23±0.16
	LB	0.354±0.00	487.1±0.02	9.68±0.08
	LPR	0.392±0.01	247.8±0.00	9.10±0.01
	LC	0.451±0.01	254.6±0.01	9.20±0.01

**NR – not reached*

Starter LAB also displayed high acidifying activity and successfully dropped the pH below 4.3 after the 6th hour of fermentation at 30°C (Fig5). It is widely accepted that higher fermentation temperatures favour the acidification process because of the increased production of lactic acid (De Vuyst et al., 2014). Indeed, the concentrations of lactic acid found in samples fermented at 30°C were up to four times higher compared with sourdoughs fermented at 20°C, whereas we observed only slight changes in the amount of acetic acid (Table 5).

Cultivation at 20°C drastically changed the performance of potential starter bacteria. The maximum specific growth rates of *Lb. plantarum* M30I–1, *Lb. paralimentarius* M30I–3 and *Lb. crustorum* M30I–9 strains decreased up to four times compared with the rates achieved during cultivation at 30°C. *Lb. brevis* M30I–2 was the only strain whose maximum specific growth rate was significantly less affected by a decrease in cultivation temperature (Table 4). The

lower fermentation temperature had a negative effect on acidifying activity of the bacteria we tested. Thus, only *Lb. plantarum* M30I–1 and *Lb. brevis* M30I–2 were active enough to drop the pH of the fermented doughs below 4.3 by the end of the fermentation cycle (Table 5). This pH is considered a benchmark of a successfully developed fermentation process (Corsetti, 2013).

Table 5. Metabolite production of Lb. plantarum M30I–1(LP), Lb. brevis M30I–2 (LB), Lb. paralimentarius M30I–3 (LPR) and Lb. crustorum M30I–9 (LC) during cultivation at 20 and 30°C.

Temperature	Starter	pH	TTA	Lactate, mmol gDM^{-1}	Acetate, mmol gDM^{-1}
		Mean ± STDEV			
20°C	**LP**	4,12±0.04	11.9±0.33	111.91±0.12	22.68±0.30
	LB	4.14±0.05	12.6±0.22	103.38±0.00	102.17±0.40
	LPR	4.40±0.02	9.05±0.22	82.01±0.33	ND*
	LC	4.42±0.02	8.93±0.45	50.13±0.12	27.17±0.20
30°C	**LP**	3.62±0.04	18.16±0.50	260.68±0.09	26.40±0.22
	LB	3.93±0.07	20.63±0.30	191.93±0.50	109.95±0.22
	LPR	3.68±0.14	17.02±0.97	230.65±0.17	25.82±0.33
	LC	3.74±0.06	17.10±0.20	233.44±0.33	26.42±0.33

**ND – not detected*

It has been shown that microorganisms growing in the transition between the exponential and stationary growth phases are preferable for sourdough propagation due to their higher cell counts and acidifying activity (Gaggiano et al. 2006). Among all tested LAB strains, only *Lb. brevis* M30I–2 was able to end exponential growth phase within 12h when inoculated within doughs fermented at 20°C (Fig.5). The high activity of *Lb. brevis* M30I–2 at 20°C could explain the predominance of this strain in the microbial population of spontaneously started Type I rye sourdoughs described in Publication I.

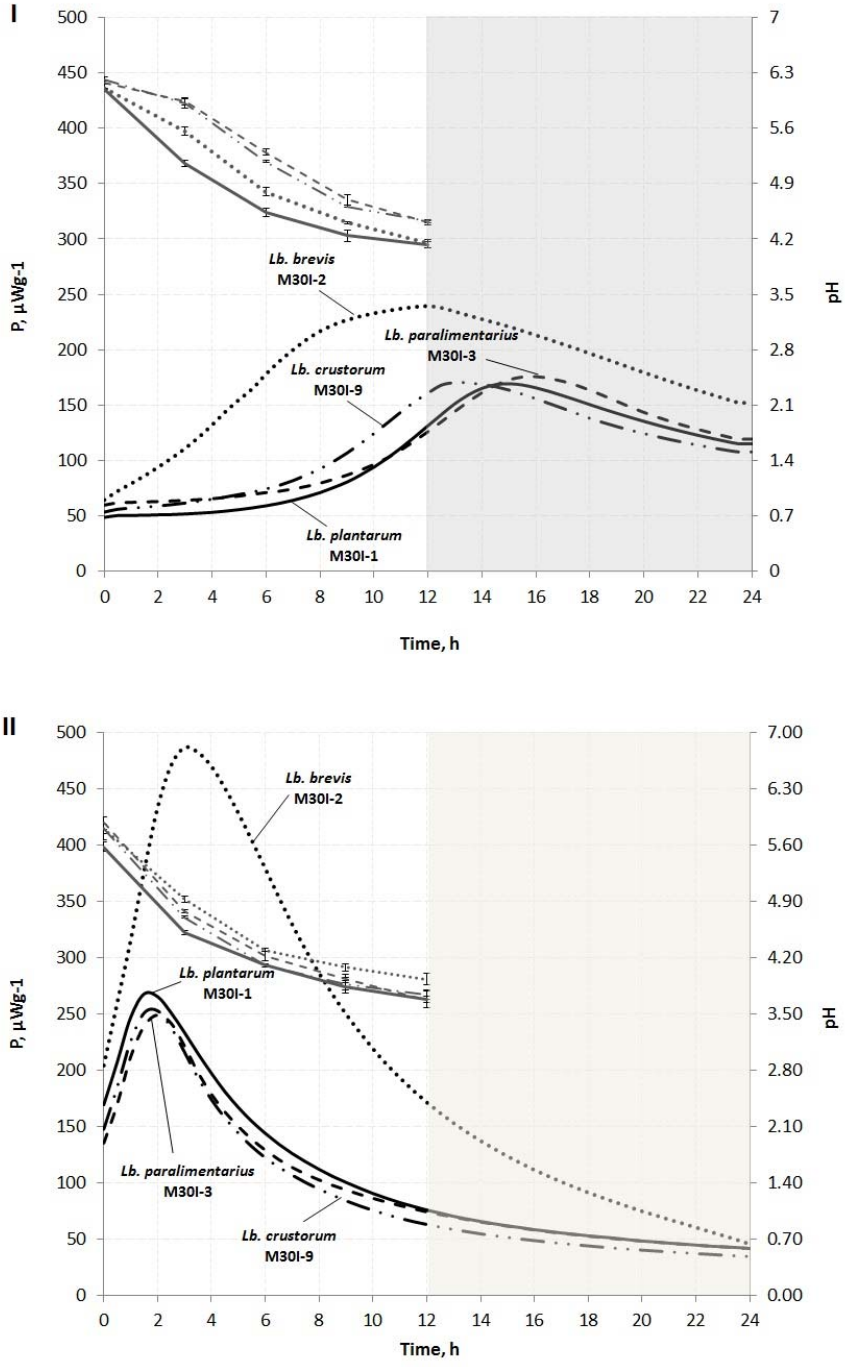

Figure 5. pH change (grey lines) and power time curves (black lines) describing the growth of individual starter bacteria in rye sourdough at 20°C (I)and 30°C (II).

5.2.4 Characterization of volatile compounds produced by selected strains during fermentation of Type I sourdough

Organoleptic properties, especially taste and aroma, still remain to be the main factors that define customer preferences when it comes to choosing rye bread (Salim–ur–Rehman et. al., 2006). It is widely accepted that the baking process defines the final aroma of the crust, with its typical roasty note, whereas the sensory properties of the crumb are developed mainly due to the activity of microorganisms during dough fermentation (Corsetti and Settanni, 2007; De Vuyst et al., 2016). In this way, the repeatability of the aroma profile under unstable propagation conditions should be taken into account during the selection of potential starters for the baking industry. Within the LAB tested in our study *Lb. plantarum* M30I–1, *Lb. paralimentarius* M30I–3 and *Lb. crustorum* M30I–9 produced an almost identical composition of volatile compounds, whereas a unique aroma profile was found in doughs fermented by *Lb. brevis* M30I–2. However, all of the strains we tested were able to produce a stable qualitative composition of volatiles at both of propagation temperatures. Thus, six alcohols were found in sourdoughs fermented with the LAB strains we studied (Table 6) and a portion of these were previously found in traditional sourdoughs of different origins (Damiani et al., 1996; Di Cagno et al., 2014; Kaseleht et al., 2011). Most of the alcohols we identified are common within all sourdoughs we have tested. An exception was 2–Methyl–1–butanol which was not detected in samples inoculated with the *Lb. brevis* strain (Table 6).

We detected six different esters in the samples we studied. Ethyl acetate, Isoamyl acetate, Hexyl acetate, Phenylethyl acetate and Ethyl undecanoate were found in all fermented doughs, whereas no traces of Propyl decanoate were detected in sourdoughs prepared using the strain *Lb. brevis* M30I–2 (Table 6). Of all the compounds we identified, only Ethyl acetate and Hexyl acetate were previously reported as volatile compounds originating from Type I sourdoughs (Damiani et al., 1996; Kaseleht et al., 2011; Di Cagno et al., 2014).

The most considerable difference in the composition of volatile compounds within the strains we tested was found between aldehyde components. Five different aldehydes were detected in the fermented doughs and only Butyrolactone was found in all samples studied. Sourdoughs fermented by *Lb. brevis* M30I–2 demonstrated the most unique aroma profile composed from Butyrolactone, (z)–2–Decenal and (E,E)–2,4–Decadienal (Table 6). The last two compounds were found only in doughs inoculated with the *Lb. brevis* M30I–2 strain. Aldehydes are assumed to be one of the key flavor compounds in rye based baked products. Thu, (E,E)–2,4–Decadienal has been previously reported as a contributor to the overall flavor of the crumb in rye breads (Burdock, 2009; Kirchhoff and Schieberle, 2001).

Table 6. Volatile compounds detected in experimental sourdoughs after 12 hours of fermentation at 20°C and 30°C. LP – dough inoculated with Lb. plantarum M30I–1, LB – dough inoculated with Lb. brevis M30I–2, LPR – dough inoculated by Lb. paralimentarius M30I–3, LC – dough inoculated by Lb. crustorum M30I–9.

Compound	LP		LB		LPR		LC	
	20	30	20	30	20	30	20	30
Ethanol	+	+	+	+	+	+	+	+
2–Methyl–1–butanol	+	+	ND	ND	+	+	+	+
2,3–Butanediol	+	+	+	+	+	+	+	+
2–Ethyl–1–hexanol	+	+	+	+	+	+	+	+
1–Octanol	+	+	+	+	+	+	+	+
1–Dodecanol	+	+	+	+	+	+	+	+
Ethyl acetate	+	+	+	+	+	+	+	+
Isoamyl acetate	+	+	+	+	+	+	+	+
Hexyl acetate	+	+	+	+	+	+	+	+
Phenylethyl acetate	+	+	+	+	+	+	+	+
Propyl decanoate	+	+	ND	ND	+	+	+	+
Ethyl undecanoate	+	+	+	+	+	+	+	+
Butyrolactone	+	+	+	+	+	+	+	+
Benzaldehyde	+	+	ND	ND	+	+	+	+
(Z)–2–Decenal	ND	ND	+	+	ND	ND	ND	ND
(E,Z)–2,4–Decadienal	+	+	ND	ND	+	+	+	+
(E,E)–2,4–Decadienal	ND	ND	+	+	ND	ND	ND	ND
Acetic acid	+	+	+	+	+	+	+	+
Butyric acid	+	+	+	+	+	+	+	+
2–Ethylhexanoic acid	+	+	ND	ND	+	+	+	+
Heptanoic acid	+	+	+	+	+	+	+	+
Octanoic acid	+	+	+	+	+	+	+	+
Nonanoic acid	+	+	+	+	+	+	+	+
Dodecanoic acid	+	+	+	+	+	+	+	+

**ND – not detected*

5.2.5 Study the co–existence of selected LAB with maltose negative yeast species in traditional rye sourdough

"Baker's yeast free bread" is a rapidly growing trend in the market. Moreover, the use of baker's yeasts in traditional bakeries is sometimes restricted by typical technological schemes applied for making rye bread (Stolz, 2003). In these cases, the number of indigenous yeasts in the microbial consortia of sourdough should be high enough to produce a sufficient amount of gas. Thus,

it is essential to study the associations between potential starter bacteria and endogenous yeast species within Type I sourdoughs.

Table 7. TTA, pH, bacterial and yeast counts in experimental mixed sourdoughs fermented at 20 and 30°C after 14 refreshment cycles. LP – Lb. plantarum M30I–1; LB – Lb. brevis M30I–2; LPR – Lb. paralimentarius M30I–3; LC – Lb. crustorum M30I–9.

Starter		pH	TTA	LAB log(CFUg^{-1})	Yeast log(CFUg^{-1})
		Mean ± STDEV			
LP + *C. humilis*	20°C	3.82±0.03	15.67±0.10	9.30±0.50	8.11±0.17
	30°C	3.74±0.05	18.38±0.11	9.23±0.33	6.60±0.11
LB + *C. humilis*	20°C	3.85±0.09	18.67±0.18	9.48±0.17	7.60±0.34
	30°C	3.71±0.02	20.82±0.05	9.50±0.65	7.15±0.22
LPR + *C. humilis*	20°C	3.90±0.03	15.07±0.07	9.21±0.44	8.42±0.69
	30°C	3.77±0.03	17.32±0.08	9.13±0.12	7.78±0.21
LC + *C. humilis*	20°C	3.80±0.07	15.53±0.08	9.53±0.28	8.34±0.85
	30°C	3.73±0.07	18.47±0.02	9.29±0.17	7.56±0.09

We analyzed sourdoughs inoculated with mixed starter cultures and propagated during 14 cycles and found very similar pH values and bacterial counts between all samples, independent of the fermentation temperature applied (Table 7).

In doughs inoculated with *Lb. plantarum* M30I–1, *Lb. paralomentarius* M30I–3 and *Lb. crustorum* M30I–9, we found similar TTA values between batches fermented at the same temperature. These values were found to lie in the range of 15.07 – 15.67 for doughs fermented at 20°C and 17.32 – 18.47 for doughs fermented at 30°C. It is known, that the presence of fructose in the dough favors the production of acetate by obligate heterofermentative LAB (Gänzle, 2015). In rye flour the amount of fructose is low, however, in the dough it can be liberated from sucrose and fructose–oligosaccharides due to the activity of enzymes in yeast (Martinez–Anaya, 2003). Indeed, doughs fermented by *Lb. brevis* M30I–2 demonstrated the highest TTA values at the both fermentation temperatures probably, due to the high amount of acetic acid (Table 7).

In high quality mature sourdough, the optimal ratio between yeasts and LAB is considered to be about 1:100 (Gobetti et al., 1994; Ottogalli et al., 1996). In the sourdoughs we studied, this ratio remained constant between temperatures only in batches fermented with *Lb. brevis* M30I–2. In case of *Lb. plantarum* M30I–1, *Lb. paralimentarius* M30I–3, and *Lb. crustorum* M30I–9 a decrease in fermentation temperature caused an increase in the count of yeast in the doughs. In a sourdough ecosystem, lactobacilli could suppress the growth of yeasts both by outcompeting them for critical nutrients or by producing antifungal compounds (Corsetti, 2013). However, the high count of both yeast and LAB in

all sourdough experiments pointed towards a mutualistic type of interaction between the LAB strains we studied and *C. humilis* SR1.

5.2.6 Influence of single starter culture on the growth of potential pathogens and reference mould *in vitro*

The microbial spoilage of bread is considered to be one of the main factors that affect their shelf–life (Smith et al., 2004). Despite the fact that the relatively high acidity and low water activity of rye bread restrict the growth of contaminative bacteria, some cases of bacteria–related foodborne outbreaks within baked products have been reported (Smith and Simpson, 1995). In this study, we screened potential starter bacteria for their ability to suppress the growth of the food–borne pathogens: *E. coli* ATCC 25922, *B. cereus* ATCC 10876, *Y. enterocolitica* ATCC27729, and *S. aureus* ATCC25923.

All tested LAB showed inhibiting activity of moderate to high intensity against all reference foodborne pathogen, when tested in a form of active biomass (Table 8). Conversely, cell–free supernatants (CFS) obtained from the LAB strains we tested demonstrated only limited inhibiting activity (Table 8). Because no antibacterial activity was detected in neutralized CFS (data not shown), we can assume that the inhibitory effect is a result of the acidic nature of the inhibitive compounds produced by the LAB we tested.

In contrast with the relatively rare cases of bacterial contamination of sourdough breads, fungal spoilage is reported to be the major cause of microbiological spoilage of rye breads (Legan, 1993; Smith et al., 2004). A number of studies have confirmed that specific LAB strains possess anti-fungal activity (Rouse et al., 2008; Gerez et al., 2009; Ryan et al., 2011), including bacteria isolated from sourdough ecosystems (Dal Bello et al., 2007; Hassan et al., 2015). In our study, the anti–fungal activity of starter LAB was tested *in vitro* using *A. brasiliensis ATCC 16404* as a reference fungi. As a result, among all LAB we tested, only *Lb. brevis* M30I–2 was able to suppress the germination of this reference mould. Strong inhibition of mould growth was detected when *Lb. brevis* M30I–2 was tested in a form of active biomass. While weak inhibition of the growth of targeted fungi was observed in a trial with CFS obtained from an *Lb. brevis* M30I–2 strain (Table 8), however, this effect completely disappeared after neutralization of CFS (data not shown).

The results obtained suggest that the observed antifungal activity of the strain was caused by organic acids produced by *Lb. brevis* M30I–2. HPLC analysis of the sourdoughs revealed that in addition to the lactic acid produced by all LAB strains, intense production of acetic acid took place in sourdoughs fermented by *Lb. brevis* M30I–2 (Table 5). Acetic acid has a dissociation constant of 4.8. As a result, at pH values below 4, as achieved by *Lb. brevis* M30I–2 in our experiments, it is present in the sourdough in an undissociated form. This uncharged form of acid is lipid soluble and may easily diffuse inside the fungal cell membrane and dissociate inside it (Piard and Desmazeaud, 1991). The H^+

protons released acidify the cytoplasm hence inhibiting the action of endogenous enzymes and forcing the cell to waste energy for the exporting excess protons. In addition, an excess of H^+ protons can denature acid sensitive proteins within the microbial cell (Piard and Desmazeaud, 1991; Russell and Diez–Gonzalez 1998; Schnürer and Magnusson, 2005). Finally, the high levels of anions that accumulate in the cytoplasm after dissociation of acetic acid can have osmotic effects on the fungal cell (Young and Foegeding, 1993).

Table 8. Effect of live biomass (B) and cell free supernatant (CFS) on the growth of reference bacteria and mould in–vitro.

	***Lb. plantarum* M30I–1**		***Lb. brevis* M30I–2**		***Lb. paralimentarius* M30I–3**		***Lb. crustorum* M30I–9**	
	B	**CFS**	**B**	**CFS**	**B**	**CFS**	**B**	**CFS**
***E. coli* ATCC 25922**	+++	++	++	+	+++	++	++	++
***B. cereus* ATCC 10876**	+++	+	+++	+++	+++	+	+++	+
***S. aureus* ATCC 25923**	++	+	++	–	+++	+	+++	–
***Y. enterocolitica* ATCC 27729**	+++	++	+++	+	+++	++	+++	++
***A. brasiliensis* ATCC 16404**	–	–	+++	+	–	–	–	–

Antimicrobial activity was interpreted as follows: "–" – no inhibition; "+" – inhibition zone = 0.1 – 0.5cm; "++" – inhibition zone = 0.5 – 1.0cm; "+++" – inhibition zone >1.0cm

Mould suppression by different strains of *Lb. brevis* species has previously been described in a number of studies. Thus, nine different strains of *Lb. brevis* isolated from sourdoughs and brewing barely have been described as producers of proteinaceous compounds and organic acids with a wide–spectrum antifungal activity (De Muynck et al., 2004; Gerez et al., 2009; Mauch et al., 2010; Guo et al., 2011). In other study performed by Tatsadjieu, Tchikoua and Mbofung (2016) two strains of *Lb. brevis* isolated from corn samples demonstrated the ability to inhibit up to 91% of all tested fungal strains during the *in vitro* screening. The synergistic effect of organic acids and ethanol produced by heterofermentative *Lb. brevis* was assumed to be the cause of this strong inhibiting activity (Tatsadjieu, Tchikoua and Mbofung, 2016). Due to the promising results of our *in vitro* antifungal study *Lb. brevis* M30I–2 strain was selected for further studies *in situ*.

5.2.7 The ability of *Lactobacillus brevis* M30I–2 to prolong the shelf–life of rye bread

It has been reported, that the formation of antifungal compounds by LAB can vary depending on the cultivation matrix (Lavermicocca et al., 2000; Stiles et al., 2002). In order to verify the results of our *in vitro* assay, we conducted additional trials with rye breads prepared using *Lb. brevis* M30I–2 as a starter culture. As a result, we detected that *Lb. brevis* M30I–2 has the ability to inhibit spore germination and suppress the development of fungal mycelium and the sporulation process in contaminated breads (Table 9).

Table 9. The ability of Lb. brevis M30I–2 to suppress the growth of mould species specific for rye breads. S– breads contaminated with spore suspension. M–breads contaminated with mycelium.

Mould–free shelf life, days*	***Penicillium corylophilum* A111**		***Penicillium citrinum* D221**		***Aspergillus niger* B213**		***Aspergillus chevalieri* D121**		***Aspergillus tubingensis* B123**	
	S	**M**	**S**	**M**	**S**	**M**	**S**	**M**	**S**	**M**
1	–	–	–	–	–	–	–	–	–	–
2	–	+	–	–	–	+	–	–	–	+
3	–	+	–	+	–	+	–	+	–	+
4	+	++	–	+	–	++	–	+	–	+
5	++	++	–	+	–	++	–	+	–	++
7	++	++	–	+	–	++	–	+	–	++

Fungistatic activity was evaluated as follows: "–" – no visual sings of fungal growth; "+" – hyphae formation; "++" – sporulation
**Compared to control bread sample prepared with Lb. crustorum M30I-9*

Previous studies reported that different species of contaminating fungi could be affected in different ways by antifungal compounds produced by specific strains of LAB (Lavermicocca et al., 2000; Magnusson and Schnürer 2001; Gerez et al., 2009; Manini et al., 2016). Indeed, the results of our *in situ* antifungal assay revealed that the mould species we tested demonstrated different level of sensitivity. Thus, *A. chevalieri* D121 and *P. citrinum* D221 showed the highest sensitivity to compounds produced by *Lb. brevis* M30I–2, thus demonstrating an absence of any singns of mycelium development during the first two days of storage (Table 9). *Lb. brevis* M30I–2 was also able to completely inhibit spore germination in *P. citrinum* D221 and all *Aspergilli* species we tested. In contrast, spores of *P. corylophilum* A111 began to germinate already at the fourth day of storage (Table 9).

P. corylophilum is a widely known bread contaminant that demonstrats a low sensitivity to calcium propionate and sodium benzoate – chemical compounds commonly used as bread preservatives (Lund et al., 1996; Lavermicocca et al., 2000). Moreover, Lavermicocca et al (2000) has reportedon the resistance of *P.*

corylophilum against inhibiting compounds produced by antifungal LAB (*Lactobacillus alimentarius* 5Q, *Lactococcus lactis subsp. lactis* 11M and *Leuconostoc citreum* 10M) tested in the study. However, the use of *Lb. brevis* M30I–2 as a starter culture prolonged the shelf life of bread contaminated by *P. corylophilum* spore suspension by up to three days compared to control samples.

5.3 PCR–based identification of contaminating fungi isolated from rye breads obtained from five different Estonian bakeries (Publication IV)

Fungal spoilage is the main factor that limits the shelf life of rye bread. Thus, around 5% of the total bread produced yearly worldwide is lost due to fungal deterioration. Fungal spores are killed by heat treatment during the baking process.However, contamination still occurs during the post baking through the air and by a contact with equipment during cooling, slicing, and packaging (Legan, 1993). Surprisingly, there is a lack of scientific information regarding the mycobiota that contaminate rye breads. Moreover, all published papers are based only on morphological identification of fungal isolates (Dich et al., 1979; Spicher, 1985; Lund et al., 1996). However, recent studies have revealed that phenotypic–based classification of closely–related mould species can be confusing and lead to misidentification (Jang et al., 2012, Silva et al., 2007; Samson et al., 2011). In our study we utilized sequencing–based molecular techniques for the fingerprinting and identification of the fungi responsible for microbiological degradation of rye breads obtained from five Estonian bakeries (referred as A_{bakery}, B_{bakery}, C_{bakery}, D_{bakery} and E_{bakery}).

Fungal biodiversity in the breads we studied drastically vary from bakery to bakery. Thus, two fungal morphotypes were detected in breads from A_{bakery} (A111, A1, Fig. 6), three in breads from B_{bakery} (B111, B123, B213 Fig. 6) and C_{bakery} (C111, C112 and C121 Fig. 6). The most morphologically diverse fungal community was observed on breads from D_{bakery} (D131, D121, D221, D231, Fig. 6), whereas in breads, obtained from E_{bakery} only one type of fungi was detected (E1, Fig. 6). It has been noted that the number of contaminative fungal species is related to the frequency of fungal infections in bakeries (Lund et al., 1996). Indeed, in our study we found a direct correlation between the diversity of isolated species and the shelf–life of rye bread. Samples from A_{bakery} and E_{bakery}, where only yeasts or one mould species was detected, had the longest shelf–life among all bread samples evaluated in this study. Conversely, breads from B_{bakery} and D_{bakery} were covered with mould colonies that began to spread already by the end of the first week of storage (Table 10).

In total, 50 mould colonies were isolated from the breads we studied. Based on the results of PCR–fingerprinting, we clustered the isolated fungi into eight RAPD groups (data not shown). Some of these were bakery–specific (RAPD II, RAPD VI – RAPD VIII in Table 10), whereas representatives of other groups were isolated from breads obtained from different bakeries.

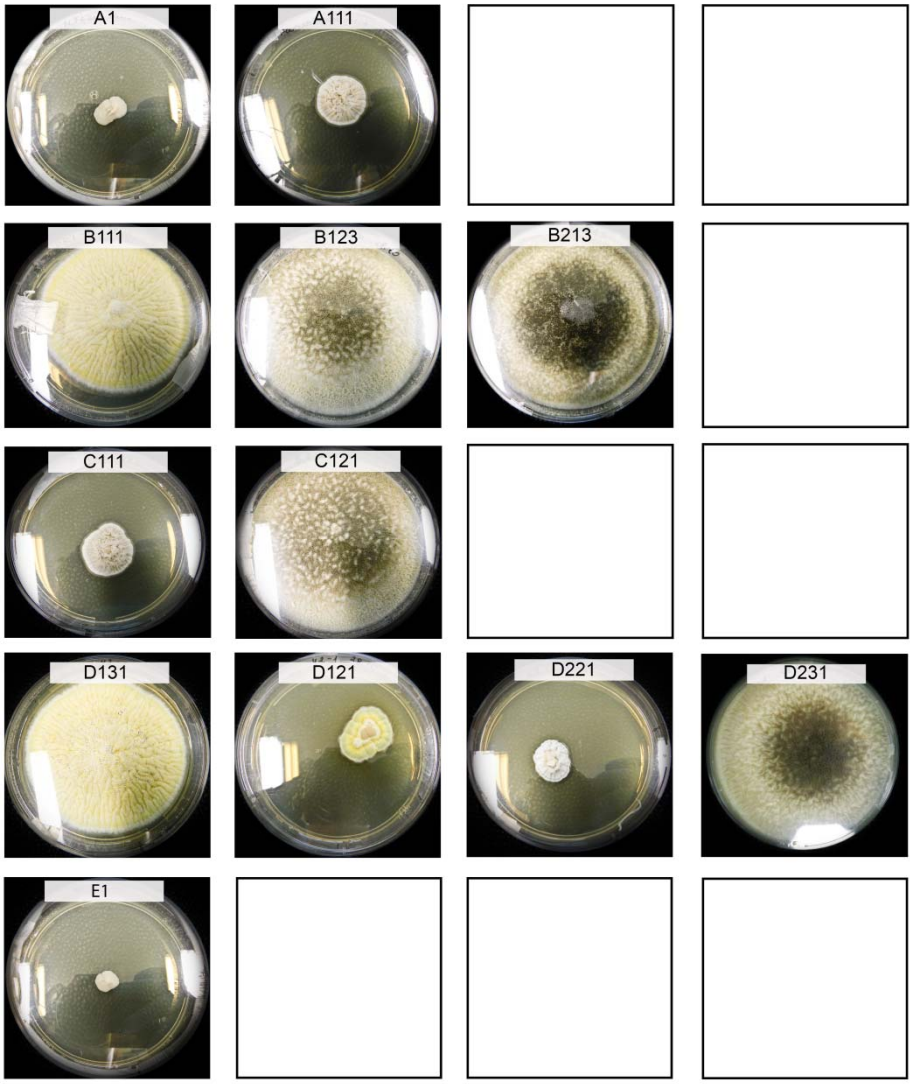

Figure 6. Fungal morphotypes obtained from breads originating from A_{bakery} (A1; A111); B_{bakery} (B111; B123; B213); C_{bakery} (C111; C121); D_{bakery} (D131; D121; D221; D231) and E_{bakery} (E1).

The representative isolates of different RAPD types were identified based on sequences within either their ITS region, the β–tubulin gene, or the D1/D2 region of the large subunit of the 28S rDNA as *A. chevalieri*, *A. flavus/oryzae*, *A. niger*, *A. tubingensis*, *P. citrinum*, *P. corylophilum*, *S. cerevisiae* and *Wickerhamomyces anomalus* (synonym *P. anomala*) species.

Table 10. Identified fungal species in bread samples from five Estonian bakeries (A, B, C, D and E) and the duration of mould–free shelf life.

Representatives of isolated fungal strains	**RAPD pattern**	**Presence in fungal population of each bakery**				
		A	B	C	D	E
P. corylophilum A111, C111	RAPD I	+	–	+	–	–
W. anomalus A1	RAPD II	+	–	–	–	–
A. niger B213, D231	RAPD III	–	+	–	+	–
A. flavus/oryzae B111, D131	RAPD IV	–	+	–	+	–
A. tubingensis B123, C121	RAPD V	–	+	+	–	–
A.chevalieri D121	RAPD VI	–	–	–	+	–
S. cerevisiae E1	RAPD VII	–	–	–	–	+
P. citrinum D221	RAPD VIII	–	–	–	+	–
Mould–free shelf life, weeks		< 3	< 1	< 1	< 1	> 3

However, molecular identification of the fungi within some of the RAPD groups was rather complicated. For example, representatives within RAPD I group where we sequenced both the β–tubulin gene and ITS region yielded 100% identical sequence data for *P. corylophilum*/*Penicillium obscurum* or *P. corylophilum/P. obscurum /Penicillium chloroleucon* respectively. All species identified belong to the *Penicillium* section *Exilicaulis*. However, as the result of the recent revision of this section by Visagie et al (2016), both *P. obscurum* and *P. chloroleucon* were considered as synonyms for *P. corylophilum* species. Thus, RAPD I isolates can be identified as the *P. corylophilum* – mould species, widely found in cereals and damp buildings (Magan, Arroyo and Alfred, 2003; McMullin, Nsiama and Miller, 2014) In a study provided by Lund et al (1996), this mould has been reported as the dominant species that contaminates rye breads made without the addition of chemical preservatives. On the other hand, another species of genera *Penicillium*, represented by isolates belonging to RAPD VIII pattern was identified as *P. citrinum* by all three molecular markers used. There is no data concerning the contamination of rye bread by *P. citrinum* species. However, *P. citrinum* was found in wheat flour and bread in the USA (Bullerman and Hartung, 1973). This species is widely found in soil and plants (Houbraken and Samson, 2011), and thus it may contaminate the bakery environment via flour particles that spread through the air and also by landing on equipment used for slicing and packaging.

None of the molecular markers we used were able to identify representatives within RAPD IV group at the species level. Sequences of both the β–tubulin gene and the D1/D2 region have similar identity to sequences of both *A. flavus* and *A. oryzae* species. These closely–related species are genetically almost identical (Chang and Ehrlich, 2010; Amaike and Keller, 2011), yet have very different economic impacts. While most *A. flavus* strains are aflatoxigenic and infect preharvest and postharvest seed crops, representatives of *A. oryzae* species have been widely used for preparation of traditional fermented foods and beverages. Genome sequence data supports the view that *A. flavus* and *A. oryzae*

are the same species with the latter representing a domesticated clade of *A. flavus* (Amaike and Keller, 2011). Although Nikkuni et al. (1998) showed that these two species could be distinguished based on ITS region sequence, Jang et al. (2012) reported that sequences of all targeted regions (ITS, D1/D2 region and β–tubulin gene) were not variable enough to distinguish *A. flavus* from *A. oryzae*.

In contrast, representatives from the RAPD III, RAPD V, and RAPD VI patterns that also belong to the *Aspergillus* genera were identified at a species level based only on their β–tubulin gene sequence, including the closely–related black–spore *Aspergilli* species *A. tubingensis* (RAPD V) and *A. niger* (RAPD III). The latter are the most difficult groups to identify using morphology–based methods (Varga et al., 2000; Varga, Frisvad & Samson, 2011; Jang et al., 2012). Whereas *A. niger* is frequently reported as a bread contaminant (Legan, 1993; Lund et al., 1996; Saranraj & Geetha, 2012), there is no data regarding the contamination of rye bread by *A. tubingensis*. The RAPD VI pattern was composed of *A. chevalieri*, a xerophilic mould growing on food with water activity down to 0.65, such as rolled oats, chocolate, some dried fruits, and nuts (Pomeranz, 1991). Its telemorph, *Eurotium chevalier*, has been detected as a contaminant within milk bread rolls (Le Lay et al., 2016).

Isolated morphotypes A1 and E1, which were microscopically identified as yeasts, clustered into RAPD II and RAPD VII patterns, respectively (Table 3). Representatives within the RAPD II pattern were identified as *W. anomalus* based on sequences of ITS and D1/D2 regions while amplification of the β–tubulin gene repeatedly failed. All three selected primer pairs performed equally well for the identification of RAPD VII as *S. cerevisiae*. In a study performed by Lund et al. (1996) a significant part of the fungi isolated from spoiled rye breads belong to yeast species that cause surface spoilage of baked products known as 'chalk moulds'. In our trial, yeasts were isolated only from samples obtained from A_{bakery} and E_{bakery}. Whereas only a single case of contamination by *S. cerevisiae* has previously been described (Spicher, 1985), *W.anomalus,* together with *E. fibuliger* and *H. burtonii* yeast species, belongs to the most frequently reported cause of 'chalk mould' bread defect (Lund et al., 1996, Deschuyffeleer et al., 2011).

Although the ITS region is considered as the universal barcode for fungal identification (Schoch et al., 2012) in our study its discriminative capacity was insufficient to identify most fungal isolates from the *Aspergillus* genera. Garnier and co–authors (2017) noted its limited taxonomic resolution for *Penicillium* and *Cladosporium* species. Thus, the β–tubulin gene should be recommended as a primary molecular marker for the identification of fungi associated with rye breads. Preliminary clustering of fungal isolates with RAPD–PCR appears to be an efficient way to reduce the expense of sequencing.

6. CONCLUSIONS

The main aim of the study was to select functional LAB strains for application in the production of Type I rye sourdough. In order to select robust authochthonous strains, we utilized technological parameters that affect the stability of the microbial population in industrial rye sourdoughs as selective factor in a long term lab–scale evolution experiment. The dominant strains were then isolated and characterized. In addition, we studied the mycoflora responsible for the spoilage of Estonian rye breads and isolated mould strains. We used these to test the ability of selected LAB strains to prolong the shelf–life of rye breads. The results of these studies can be summarized as follows:

I. Maintaining a stable fermentation temperature is crucial for the stability of microbial community in industrial Type I sourdoughs. Fluctuations of fermentation temperature cause quantitative and qualitative changes in microbial consortia of rye sourdough which can lead to fermentation failure.

II. Two months of daily propagation of spontaneously started rye sourdough at 20°C and 30°C resulted in the selection of four LAB strains adapted to Type I rye sourdough ecosystems. Among them, *Lactobacillus brevis* M30I–2 demonstrated the highest potential as a starter bacteria for Type I rye sourdoughs due to its rapid growth and fast acidification at ambient fermentation temperatures. Moreover, *Lactobacillus brevis* M30I–2 displayed an ability to prolong the shelf–life of rye breads contaminated by the mould species responsible for the fungal spoilage of Estonian rye breads.

III. Mould species from *Aspergillus* and *Penicillium* appeared to be the main cause of fungal infection in rye breads from Estonian bakeries. We recommend that the β–tubulin gene should be used as a primary molecular marker for the identification of fungi associated with rye breads.

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SUMMARY

Sourdough fermentation has a significant impact on the quality of rye bread. A lack of gluten and the presence of active α – amylase in rye flour makes rye dough extremely sensitive to the acidification rate. Moreover, metabolites produced by microorganisms during fermentation play a crucial role in the formation of classic rye bread aromas and help in the biopreservation of the end product. As with other living organisms, lactic acid bacteria require a certain environmental conditions for successful growth and metabolite production. In most large–scale bakeries, fermentation is controlled by industrial equipment that ensures consistent production of high–quality end products. Yet, many artisan bakeries still use traditional (Type I) sourdough propagation technologies and face seasonal fluctuations in microbial composition that are mainly caused by uncontrolled fermentation conditions.

The main aim of this work was to select a robust, metabolically active and functional LAB starter for the further use in Type I sourdough processes. For this we (i) determined which technological parameters critically affect the stability of industrial Type I rye sourdoughs; (ii) applied these as selective factors in evolution experiments to find robust LAB strains; and (iii) isolated and characterized dominant LAB strains. In addition, biodiversity of fungi responsible for microbiological spoilage of rye bread was evaluated.

Our study confirmed that maintaining a constant fermentation temperature is critically important in achieving a stable microbial population in industrial sourdoughs. Thus, the seasonal fluctuations in the small-scale bakeries we studied were driven by changes in fermentation temperature which resulted in unstable chemical and microbiological composition within their sourdoughs. This, in turn, caused fermentation failures and decreased the quality of their end products.

We performed two parallel evolution experiments at two different fermentation temperatures (20 and 30ºC). This selective factor resulted in four dominant LAB strains: *Lactobacillus brevis* M30I–2 *Lactobacillus plantarum* M30I–1, *Lactobacillus paralimentarius* M30I–3, and *Lactobacillus crustorum* M30I–9. Dominant lactobacilli were isolated and characterized to evaluate their potential as starter culture strains for the production of traditional sourdoughs. Among all strains tested, *Lactobacillus brevis* M30I–2 demonstrated the highest potential for application in Type I rye sourdough fermentations due to its (i) rapid growth and production of technologically valuable metabolites at ambient fermentation temperatures (ii); ability to maintain the number of maltose–negative yeasts during co–cultivation; (iii) significant inhibiting effect on the growth of contaminating fungi which prolonged the shelf–life of breads produced from it.

The fungi we isolated and identified in rye bread mainly belong to *Aspergillus* and *Penicillium* species. Comparison of the performance of three different molecular markers, the ITS region, the D1/D2 region of 28S rRNA, and

the β–tubulin gene, revealed that the latter has the highest taxonomic resolution. Sequencing of the β–tubulin gene allowed us to distinguish *Aspergillus tubingensis* from its close relative – *Aspergillus niger*, thus supplementing the list of moulds related to mycological spoilage of rye breads.

KOKKUVÕTE

Taigna kääritamine avaldab märkimisväärset mõju rukkileiva kvaliteedile. Aktiivse α–amülaasi olemasolu ja madal gluteenisisaldus rukkijahus teevad rukkitaigna happesuse suhtes eriti tundlikuks. Metaboliidid, mida toodavad juuretise piimhappebakterid ja pärmid kääritamisprotsessi käigus, mängivad olulist rolli klassikalise rukkileiva aroomi kujunemisel ning lõpptoote säilimisel. Nagu iga elusorganism, vajavad juuretise piimhappebakterid teatud keskkonnatingimusi edukaks kasvuks ja metaboliitide tootmiseks. Standardiseeritud ja kvaliteetsete lõpptoodete saamiseks kontrollitakse enamikes suurtes leivatööstustes juuretise kääritamisprotsessi vastavate seadmetega. Samas kasutavad mõned väiksed leivatööstused siiamaani traditsioonilist (I tüüpi) juuretise uuendamise tehnoloogiat. See tehnoloogia toob endaga kaasa hooajast sõltuvaid kõikumisi juuretise mikroobioloogilises koostises kontrollimatute fermentatsioonitingimuste tõttu.

Antud töö peamine eesmärk oli selekteerida välja stabiilne, metaboolselt aktiivne ja funktsionaalne piimhappebakterite starterkultuur I tüüpi juuretise tootmiseks. Selle eesmärgi saavutamiseks: (i) määrati kriitilised tehnoloogilised parameetrid, mis mõjutavad tööstusliku I tüüpi rukkijuuretise stabiilsust; (ii) kasutati neid parameetreid vastupidavate piimhappebakteritüvede selekteerimiseks; (iii) isoleeriti ning iseloomustati dominantsed tüved. Lisaks uuriti rukkileiva mikrobioloogilise riknemise eest vastutavate hallituste mitmekesisust.

Käesolevas uuringus tuvastati püsiva kääritamistemperatuuri märkimisväärset rolli stabiilse mikrobioloogilise populatsiooni saavutamises tööstuslikus juuretises. Hooajalised kõikumised fermentatsioonitemperatuuris uuritavates väikestes leivatööstustes väljendusid juuretise keemilise ja mikrobioloogilise koostise ebastabiilsuses. See omakorda põhjustas fermentatsiooni ebaõnnestumist ning alandas lõpptoote kvaliteeti.

Evolutsioonieksperimendi tulemusena valiti neli dominantset piimhappebakteri tüve: *Lactobacillus brevis* M30I–2 *Lactobacillus plantarum* M30I–1, *Lactobacillus paralimentarius* M30I–3 ja *Lactobacillus crustorum* M30I–9. Selektiivse faktorina kasutati erinevaid kääritamistemperatuure (20 ja 30 ° C). Domineerivad laktobatsillid eraldati ning neid kirjeldati, et hinnata nende potentsiaalset rakendust traditsiooniliste (I tüüpi) rukkijuuretiste tootmiseks. Testitud tüvede vahel näitas *Lb. brevis* M30I–2 kõige suuremat potentsiaali I tüüpi rukkijuuretiste starterkultuurina tänu oma: (i) kiirele kasvule ja tehnoloogiliselt väärtuslike metaboliitide tootmisele (ii); võimele toetada maltoos-negatiivsete pärmide kasvu traditsioonilistes juuretistes; (iii) märkimisväärsele inhibeerivale mõjule hallitusseente kasvule ning võimalusele pikendada rukkileiva säilivusaega.

Antud uuringus isoleeritud ja identifitseeritud rukkileiva saastumist põhjustavad hallitused kuulusid peamiselt *Aspergillus* ja *Penicillum* perekonda. Kolme erineva molekulaarse markeri (ITS regiooni D1/D2 piirkonna, 28S rRNA

ja β–tubuliini geeni) võrdlus näitas, et β–tubuliin geenil on kõige parem taksonoomiline eraldusvõime. Nii võimaldas β-tubuliini geeni sekveneerimine meil eristada *Aspergillus tubingensis*'t tema lähisugulasest - *Aspergillus niger* tüvest, täiendades sellega rukkileiva riknemisega seotud hallitusseente nimekirja.

CURRICULUM VITAE

1. Personal data

Name	Marianna Surženko (Bessmeltseva)
Date of birth	26.08.1985
Place of birth	Tallinn, Estonia
Citizenship	Estonian

2. Contact information

E-mail	marianna@tftak.eu
Address	AS TFTAK, Akadeemia tee 15a, Tallinn 12618, Estonia
Contact phone	+37255650962

3. Education

2011– present	Tallinn University of Technology, Department of Chemistry and Biotechnology, doctoral studies
2008–2011	Tallinn University of Technology, Faculty of Chemicals and Materials Technology, MSc in Food Engineering and Product Development
2004–2008	Tallinn University of Technology, Faculty of Chemicals and Materials Technology, BSc in Food Engineering and Product Development
1992–2004	Tallinn 53th Secondary School

4. Professional Employment

2015 – present	Lallemand Bio Inc. Bio-Ingredients division; Researcher
2009 – 2015	Center of Food and FermentationTechnologies; Researcher

5. Special courses

01.02–01.06.2014	Research work in BIOR (Institute of Food Safety, Animal Health and Environment). Riga, Latvia

.

6. Defended thesis

Master thesis	Supervisor Dr. Inga Sarand. Title: “Technological properties of lactic acid bacteria isolated from industrial rye sourdough”

7. Supervised Theses

2012	Co - supervised Bachelor thesis of Ksenia Kuksina "Selection of bacteria starters for small-scale bakeries"
2014	Supervised master thesis of Marju Seer "Isolation and identification of bread-contaminating fungi"

8. Awards

2012	Best poster award at the "Cereal Fermentation for Future Foods", Helsinki, Finland

ELULOOKIRJELDUS

1. Isikuandmed

Nimi	Marianna Surženko (Bessmeltseva)
Sünniaeg	26.08.1985
Sünnikoht	Tallinn, Eesti
Kodakondsus	Eesti

2. Kontaktandmed

E-mail	marianna@tftak.eu
Address	AS TFTAK, Akadeemia tee 15a, Tallinn 12618, Estonia
Telefon	+37255650962

3. Hariduskäik

2011– …	Tallinna Tehnikaülikool, Keemia ja biotehnoloogia instituut, doktoriõpe
2008–2011	Tallinna Tehnikaülikool, Keemia – ja materjalitehnoloogia teaduskond, Toidutehnika ja tootearenduse magistriõpe (MSc)
2004–2008	Tallinna Tehnikaülikool, Keemia – ja materjalitehnoloogia teaduskond, Toidutehnika ja tootearenduse bakalaureuseõpe (BSc)
1992–2004	Tallinna 53. keskkool

4. Teenistuskäik

2015 – present	Lallemand Bio Inc. Teadur
2009 – 2015	Toidu– ja Fermentatsioonitehnoloogia Arenduskeskus; Teadur

5. Täiendõpe

01.02–01.06.2014	Välispraktikum BIOR-is (Institute of Food Safety, Animal Health and Environment). Riia, Läti.

6. Kaitstud lõputööd

Magistritöö	Juhendaja Dr. Inga Sarand, “Tööstuslikust haputaignast isoleeritud piimhappebakterite tehnoloogiliste omaduste iseloomustamine”

7. Juhendatud lõputööd

2012	Ksenia Kuksina, bakalaureusekraad "Starterkultuuride valik väikestele leivatööstusele"
2014	Marju Seer magistritöö "Leivahallituste isoleerimine ja identifitseerimine"

8. Tunnustused

2012	Teadustöö auhind (Cereal Fermentation for Future Foods" Helsingis, Soome)

PUBLICATION I

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RESEARCH ARTICLE

Diversity and Stability of Lactic Acid Bacteria in Rye Sourdoughs of Four Bakeries with Different Propagation Parameters

Ene Viiard[1,2]*, Marianna Bessmeltseva[1,2], Jaak Simm[3], Tiina Talve[1], Anu Aaspõllu[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

* ene@tftak.eu

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Abstract

We identified the lactic acid bacteria within rye sourdoughs and starters from four bakeries with different propagation parameters and tracked their dynamics for between 5–28 months after renewal. Evaluation of bacterial communities was performed using plating, denaturing gradient gel electrophoresis, and pyrosequencing of 16S rRNA gene amplicons. *Lactobacillus amylovorus* and *Lactobacillus frumenti* or *Lactobacillus helveticus*, *Lactobacillus pontis* and *Lactobacillus panis* prevailed in sourdoughs propagated at higher temperature, while ambient temperature combined with a short fermentation cycle selected for *Lactobacillus sanfranciscensis*, *Lactobacillus pontis*, and *Lactobacillus zymae* or *Lactobacillus helveticus*, *Lactobacillus pontis* and *Lactobacillus zymae*. The ratio of species in bakeries employing room-temperature propagation displayed a seasonal dependence. Introduction of different and controlled propagation parameters at one bakery (higher fermentation temperature, reduced inoculum size, and extended fermentation time) resulted in stabilization of the microbial community with an increased proportion of *L*. *helveticus* and *L*. *pontis*. Despite these new propagation parameters no new species were detected.

Introduction

Sourdough is a mixture of flour and water that is fermented with lactic acid bacteria (LAB) and yeasts. Traditional sourdoughs are propagated by backslopping over many decades by mixing a portion of mature sourdough with fresh flour and water and fermenting this into a new batch of sourdough [1, 2]. In mature sourdough, both homo- and hetero-fermentative species of LAB are prevalent and the community is typically dominated by members from the genus *Lactobacillus* [3].

Over 50 different species of LAB have been isolated from sourdoughs of different origin [4]. Despite this large number of identified species, mature sourdoughs typically contain only two or three dominant species. *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus sanfranciscensis*, and *Lactobacillus acidophilus* [1, 5–6] species are most often encountered in rye sourdoughs, which are used to make rye bread, a staple of the Nordic diet.

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Sourdoughs can be classified into three types according to the technology used for their production [1, 7–8]. Type I sourdoughs are produced using a traditional method based on daily renewal. Type II sourdoughs used in large-scale production are semi-fluid and have good handling properties. Long term continuous propagations are common in type II sourdough processes. Type III sourdoughs are generally 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 with a starter culture [2]. The baking industry currently tends to begin sourdough fermentation with defined commercial starter cultures with specific properties [1]. Unfortunately, those strains may not adapt to the sourdough propagation conditions in the bakery and are often not competitive enough in the long term to fight off LAB that enter the process from either raw materials or the bakery environment. Therefore, to maintain a desirable microbial community, the sourdough cycle is frequently restarted [9–10]. The microbial composition of sourdoughs is affected by the process technology and applied conditions: fermentation temperature and time, inoculum size, water content (characterized by dough yield), production environment, and type of flour [1, 11–13].

Information regarding the composition and stability of sourdoughs used in industrial-scale production is limited. The traditions of sourdough preparation and bread making are region-dependent, which influences the sensory characteristics chosen for industrially prepared breads [14]. The aims of this work are i) to compare LAB communities in sourdoughs that originate from bakeries that apply different sourdough propagation parameters 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. Both culture dependent and culture independent methods were used to characterize the microbial communities within the sourdough samples.

Materials and Methods

Sourdough samples from bakeries

The sourdoughs studied originate from four bakeries that use flour of the same origin (rye flour type 1370, extraction rate 85%, Tartu Mill AS, Estonia) and are referred to as A_{bakery}, B_{bakery}, C_{bakery}, and D_{bakery}. The bakeries use different sourdough propagation parameters (presented in Table 1); two sourdoughs are type II (A_{bakery} and B_{bakery}) and two are type I (C_{bakery} and D_{bakery}). 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. The temperature of each D_{bakery} sourdough sample is provided in Table 1.

The sourdough process in A_{bakery} was initiated with a freeze-dried sourdough made from a mature sourdough produced six years previously at the same bakery (sample A0). B_{bakery} sourdough had been successfully propagated for three years (sample Bs) and was then renewed from fresh cooled sourdough sourced from another bakery belonging to the same corporation (sample B0). C_{bakery} sourdough fermentation was initiated with a freeze-dried commercial starter (C0) one year before the first sourdough sample was collected. D_{bakery} sourdough was initiated in the 1980's from a commercial starter. During the course of this study D_{bakery} adopted new propagation parameters with a well-controlled fermentation temperature in an attempt to improve both the stability of the sourdough and optimize the sourdough production cycle (Table 1). 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 33% to 10%. Sourdough sample D4 was taken before adopting the new sourdough propagation cycle. Sample D5 was collected from sourdough that had been propagated for five months after the

Table 1. Sourdough propagation parameters (fermentation temperature and time, inoculum size, dough yield), starter used to initiate fermentation, and sampling schedule in four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}). In C_{bakery} and D_{bakery} the fermentation was carried out at room temperature (RT). 'Months' indicate time passed from the beginning of a new sourdough cycle (A_{bakery}, B_{bakery}, and C_{bakery}) or from the collection of the first sourdough sample (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)
Inoculum size	10%	6%	~ 33%	~ 33%
Fermentation time	10 h	16 h	~ 4 h	~ 4 h
Dough yield	250	400	~ 200	~ 200
Starter	Freeze-dried sourdough culture	Active sourdough starter	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 (ambient temperature 19°C)
	A1–1.2 months	B0 –fresh sourdough starter	C1–12 months	D2–3 months (30°C)
	A2–3.5 months	B1–0.25 months	C2–21 months	D3–5 months (28°C)
	A3–4.5 months	B2–1 month	C3–28 months	D4–19 months (23°C, before transfer to 30°C)
	A4–8.5 months	B3–2.5 months		D5–24 months (5 months after transfer to 30°C)
		B4–6 months		

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upgrade. In addition, 18 sourdough samples were collected between these two time points, however, only pH and DGGE analysis was performed.

Chemical analysis of sourdoughs

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

Enumeration of lactic acid bacteria

The cell density of culturable LAB in each sourdough sample was determined by plate counting. 5 g of sourdough was mixed with 45 ml of sterile 0.85% NaCl solution. A series of decimal dilutions were plated on MRS agar (LabM, UK) in duplicate. In addition, sample C3 was plated on an mMRS agar (MRS with added 2% maltose; pH 5.6) and SDB agar (2% maltose; 0.03% Tween80; 0.6% trypticase; 1% yeast extract; pH 5.6) [15]. 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 lactic acid bacteria isolates and sourdough samples

Selected colonies were checked for purity by streaking. The cultures were suspended in dH_2O with a loop and subjected to DNA extraction using FTA membrane cards (Whatman Inc., USA) following the method provided by the manufacturer. Total DNA extraction from the sourdough samples was performed using 5 g of sourdough, which had been homogenized by vortexing with 45 ml of sterile physiological solution. 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. A GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, Inc., USA) was used for DNA extraction from the obtained pellet in the case of 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} [16].

Fingerprint typing of isolates with Rep-PCR

For each sourdough sample, 20 colonies were randomly picked from plates with suitable dilution (usually 20 to 200 colonies per plate) and analyzed by Rep-PCR. Rep-PCR with primer $(GTG)_5$ (all primers in this work were obtained from Microsynth, Switzerland) was performed as described by Viiard [16] 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 lactic acid bacteria 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 [17] and 16R1522 [18] followed by column purification of the amplified fragment with a GeneJET PCR Purification Kit (Thermo Scientific Inc., USA) and sequenced in a commercial facility. 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) and the results were confirmed with the Greengenes 16S rRNA gene database (Lawrence Berkeley National Laboratory, USA).

Denaturing gradient gel electrophoresis analysis of microbial communities

Denaturing gradient gel electrophoresis (DGGE) analysis was performed to monitor the dynamics of microbial communities within sourdoughs. V3 region of the bacterial 16S rRNA genes was amplified using primers F357-GC and 518R as described by Gafan and Spratt [19] to obtain approx. 160 bp fragments. Polyacrylamide gel (8% acrylamide-N,N'-methylenebisacrylamide; 37.5:1) with a gradient from 35 to 70% urea and formamide (100% corresponding to 40% 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 yeasts, the primers U1GC and U2 were used to amplify the 28S rRNA genes, as described by Meroth [20] to obtain approx. 300 bp amplicons. 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 fragments were reamplified using F357/518R or U1/U2 primer pairs for bacterial and yeast DNA, respectively, and sequenced in a commercial facility.

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 [21–22]. 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 [23] according to standard operating procedures [24]. Reads shorter than 150 bp or containing more than eight homopolymers were removed from the dataset.

Table 2. Mean values ± standard deviation of pH, total titratable acidity (TTA) and cell density of presumptive lactic acid bacteria (LAB) of rye sourdoughs from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}). Samples are coded according to the description reported in Table 1.

Sample	pH	TTA	LAB
		(ml 0.1 N NaOH / 10 g)	(log CFU g^{-1})
A0	NA*	NA	7.08 ± 0.11
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	6.95 ± 0.04
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

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Sequences were aligned to the SILVA reference 16S rRNA database [25]. 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 we calculated the rate of forming new OTUs when one sequence is added to the set of 500 sequences. The closest match to each OTU within the Greengenes 16S rRNA gene database (Lawrence Berkeley National Laboratory, USA) was identified using BLAST with a minimum of 97% similarity. 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 using Yue and Clayton distances [26] within MOTHUR and results were visualized using R software (version 3.0.3).

Nucleotide sequence accession numbers

Pyrosequencing data is available in the GenBank database under accession numbers KM972414—KM972548.

Results

Chemical analysis and LAB enumeration of rye sourdoughs

The chemical properties of A_{bakery}, B_{bakery} and C_{bakery} sourdoughs were rather stable throughout the study (Table 2). The average pH value of the sourdough throughout the study was

3.64 ± 0.08 in A_{bakery}, 3.56 ± 0.08 in B_{bakery} and 4.10 ± 0.09 in C_{bakery}. B_{bakery} sourdough was characterized by a high TTA in all samples except the initiating starter dough B0. Significant seasonal fluctuations in pH occurred in the sourdough from D_{bakery}, where the average pH value of the sourdough was 4.06 ± 0.18. Sample D1 with a pH value of 4.28 was taken in February, when temperature of the sourdough was 19°C. During warmer months June (D2) and August (D3) the pH values decreased to 3.96 and 3.86, respectively.

The cell density of LAB was high and stable in A_{bakery} (on average 8.95 ± 0.13 log CFU g^{-1}) and B_{bakery} (on average 8.67 ± 0.32 log CFU g^{-1}), with the exception of sample B1, which exhibited a lower cell density (Table 2). In case of C_{bakery} unusually low values of LAB cell density (order of magnitude: 6 log CFU g^{-1}) were obtained for samples C1 and C2 (Table 2). Two fold higher cell density was found for sample C3, but the related plates were incubated for additional 24 h compared to the samples C1 and C2. In D_{bakery} the LAB cell density (on average 8.47 ± 0.54 CFU g^{-1}) depended on the ambient temperature in the bakery and was higher during summer (samples D2 and D3).

Analysis of the LAB community in rye sourdoughs

Sourdough samples from A_{bakery} were monitored for over eight months after renewal from a freeze-dried starter (previously published by Viiard [16]). Based on the results from culture dependent analysis, the dominating LAB in the freeze-dried starter dough belonged to species *Lactobacillus helveticus*, *Lactobacillus panis* and *Lactobacillus pontis* (Table 3 and Fig 1). 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 prevalent species during over eight months of propagation (Table 3 and S1 Fig). The pyrosequencing analysis confirmed that the microbial community within the A_{bakery} sourdough was remarkably stable (Table 3 and Fig 2). Data of sequences and OTUs from 16S rRNA pyrosequencing performed using DNA extracted from sourdoughs sampled at A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery} are shown in Table 4. The trimmed amplicon length of all sourdough samples was in the range 228–262 bp. The total number of sequences before processing (raw reads) was 42,388; on average 2231 sequences per sample were obtained. After data processing (reads) in total 34,906 sequences remained.

After the first sample (Bs) was collected from B_{bakery}, 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* were dominant species within all sourdough samples collected from B_{bakery} (Table 3 and Fig 1). The ratio between this species varied during propagation. The same fingerprint type of dominant *L. amylovorus* was detected throughout the study (data not shown). *L. panis* entered the sourdough cycle with sample B0 and remained constant within the sourdough at low counts during subsequent propagation. DGGE analysis confirmed the dominance of *L. amylovorus* and *L. frumenti* (Table 3 and S1 Fig). On the contrary, *L. panis* was undetectable by both DGGE and 16S rRNA pyrosequencing (Table 3 and Fig 2). Pyrosequencing analysis revealed that *L. frumenti* and, especially, *L. amylovorus*, were the dominant OTUs in the sourdough samples collected at the B_{bakery}. Additional OTUs (*Lactobacillus secaliphilus* and *Lactobacillus* sp.) were found as sub-dominant in all the B_{bakery} samples. In C_{bakery}, *L. plantarum*, *Lactobacillus rhamnosus* and *Lactobacillus casei/paracasei* were isolated from the starter sample (C0) (Fig 1). After one year of propagation (C1), *L. casei/paracasei* persisted and other species (*Lactobacillus zymae*, *L. fermentum*, *Leuconostoc lactis* and *Pediococcus acidilactici*) were detected. In the sourdough samples taken 21 and 28 months after renewal (samples C2 and C3), all the LAB species previously detected, except for

Table 3. Bacterial species/genera found in the rye sourdough samples from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) through culture dependent analysis, DGGE or 16S pyrosequencing. Presence (+) or absence (–) of species is indicated for each sample in the following order: culture dependent analysis / DGGE / 16S pyrosequencing. N–not analyzed. Samples are coded according to the description reported in Table 1.

		Bacteroides sp.	*Flavobacteriaceae bacterium*	*Lactobacillus amylovorus*	*Lactobacillus casei/paracasei*	*Lactobacillus coleohominis*	*Lactobacillus fermentum*	*Lactobacillus frumenti*	*Lactobacillus helveticus*	*Lactobacillus panis*	*Lactobacillus plantarum*	*Lactobacillus pontis*	*Lactobacillus rhamnosus*	*Lactobacillus sanfranciscensis*	*Lactobacillus secaliphilus*	*Lactobacillus vaginalis*	*Lactobacillus zymae*	*Leuconostoc lactis*	*Pantoea sp.*	*Pediococcus acidilactici*
A_{bakery}	A0	–/–/–	–/–/–	–/–/–	–/–/–	–/–/+	–/–/–	–/–/–	+/+/+	+/+/+	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–
	A1	–/–/–	–/–/–	–/–/–	+/–/–	–/–/–	–/–/–	–/–/–	+/+/+	–/+/+	–/–/–	–/+/+	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–
	A2	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	+/+/+	+/+/+	–/–/–	–/+/+	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–
	A3	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	+/+/+	+/+/+	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	+/–/+	–/–/–	–/–/–	–/–/–	–/–/–
	A4	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	+/+/+	+/+/+	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	–/–/+	–/–/–	–/–/–	–/–/–	–/–/–
B_{bakery}	Bs	–/–/–	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/+	–/–/–	–/–/–	–/–/–	–/–/–
	B0	–/–/–	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	+/+/+	–/–/–	+/–/–	–/–/–	–/–/+	–/–/–	–/–/–	–/–/–	–/–/+	–/–/–	–/–/–	–/–/–	–/–/–
	B1	–/–/–	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	+/+/+	–/–/–	+/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/+	–/–/–	–/–/–	–/–/+	–/–/–
	B2	–/–/+	–/–/+	+/+/+	–/–/–	–/–/–	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/+	–/–/–	–/–/–	–/–/–	–/–/–
	B3	–/–/–	–/–/+	+/+/+	–/–/–	–/–/–	–/–/–	+/+/+	–/–/–	+/–/–	–/–/–	–/–/+	–/–/–	–/–/–	–/–/–	–/–/+	–/–/–	–/–/–	–/–/–	–/–/–
	B4	–/–/–	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	+/+/+	–/–/–	+/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/+	–/–/–	–/–/–	–/–/–	–/–/–
C_{bakery}	C0	–/–/–	–/–/–	–/–/–	+/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	+/–/–	–/+/+	+/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–
	C1	–/–/–	–/–/–	–/–/–	+/–/–	–/–/–	+/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/+/–	–/–/–	–/+/+	–/–/–	–/–/–	+/–/+	+/–/–	–/–/–	+/–/–
	C2	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	+/+/+	–/–/–	–/+/+	–/–/–	–/–/–	+/–/–	–/–/–	–/–/–	–/–/–
	C3	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	+/+/–	–/–/–	–/+/+	–/–/–	–/–/–	+/–/+	–/–/–	–/–/–	–/–/–
D_{bakery}	D1	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/+/–	–/–/–	–/–/–	–/+/+	–/–/–	–/–/–	–/–/–	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–
	D2	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/+/+	–/–/–	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	–/–/–	+/–/–	–/–/–	–/–/–	–/–/–
	D3	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/+/+	–/–/–	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	–/–/–	+/–/+	–/–/–	–/–/–	–/–/–
	D4	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	+/+/+	–/–/–	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	–/–/–	+/–/+	–/–/–	–/–/–	–/–/–
	D5	N/–/–	N/–/–	N/–/–	N/–/–	N/–/–	N/–/–	N/–/–	N/+/+	N/–/–	N/–/–	N/+/+	N/–/–	N/–/–	N/–/–	N/–/–	N/+/–	N/–/–	N/–/–	N/–/–

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L. zymae, seemed to be replaced by *L. pontis*. In contrast, DGGE analysis revealed *L. pontis* as the only species in sample C0 and *L. pontis* and *L. sanfranciscensis* in all the remaining sourdough samples (Table 3 and S1 Fig). Overall, pyrosequencing analysis of C_{bakery} samples was in agreement with DGGE, excluding the lack of *L. pontis* in C1 and C3 samples and the presence, at high relative abundance, of *L. zymae* in C1 (Table 3 and Fig 2). Given the discrepancy between culture dependent and independent analyses regarding the presence of *L. sanfranciscensis* in the samples collected at the C_{bakery}, the C3 sample was analyzed using two additional media, SDB and mMRS. *L. sanfranciscensis* could be isolated after an extended incubation time (72 h) of mMRS plates (data not shown).

The sourdough samples from D_{bakery} contained *L. zymae*, *L. pontis* and *L. helveticus* (Figs 1 and 2 and S1 Fig). The relative proportion of these species in a given sourdough sample depended 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 (D2, D3, and D4) collected during warmer periods.

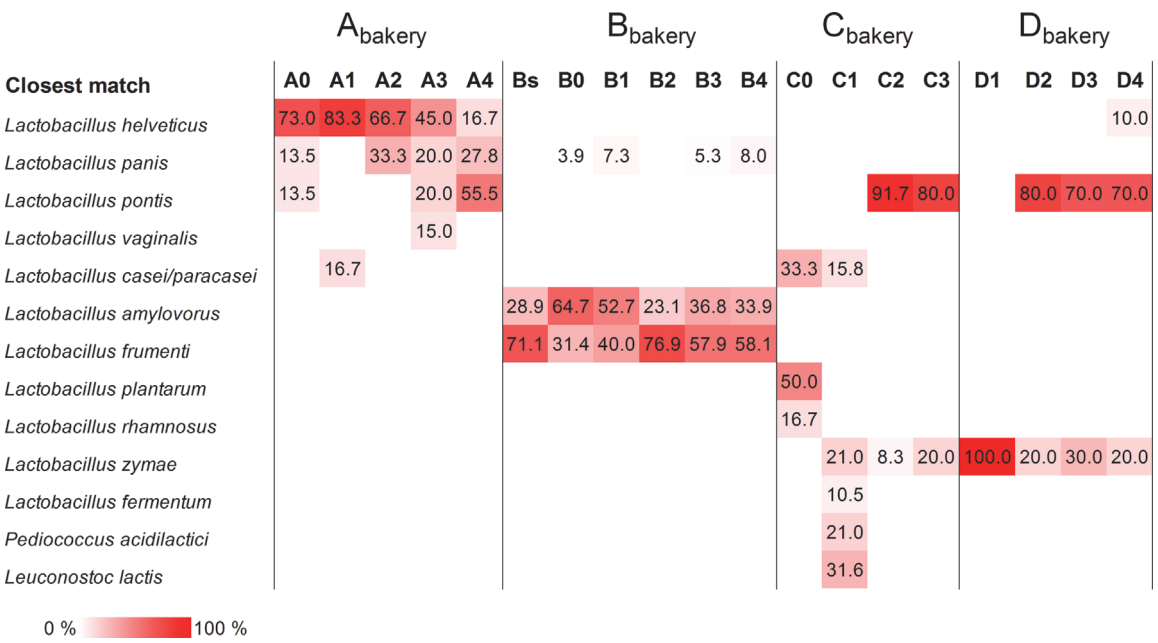

Fig 1. Composition of lactic acid bacterium species, expressed in percentage of the total number of isolates, in rye sourdoughs from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}). Samples are coded according to the description reported in Table 1.

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Impact of new propagation parameters on the LAB community of D_{bakery} sourdough

In order to improve the stability of D_{bakery} sourdough, a new sourdough propagation protocol was applied with a controlled fermentation temperature, prolonged fermentation time and reduced inoculum size. As ascertained through culture independent analyses, *L. pontis* and *L. helveticus* species dominated in the sourdough (sample D5) after 5 months of propagation performed under the new protocol (Figs 2 and 3). The pH of the sourdough ranged from 3.73–3.79 during the five months of observation (Fig 3).

Analysis of yeast communities in rye sourdoughs

DGGE analysis of amplified 28S rRNA (S2 Fig) was performed to identify the yeast species in the sourdoughs collected at the four bakeries. *Kazachstania telluris* was the only yeast species harbored in the freeze-dried starter (A0), as well as in all the sourdough samples collected at the A_{bakery}. No yeast species were detected in B_{bakery} sourdough. *Candida humilis* was the only yeast species detected in C_{bakery} and D_{bakery} sourdough samples, whereas no yeast species were detected in the starter (C0) used in C_{bakery} (S2 Fig).

Beta-diversity across the sourdough samples

Beta-diversity analysis was performed on the pyrosequencing data to compare diversity between each sourdough sample and to determine the similarity (or difference) in species

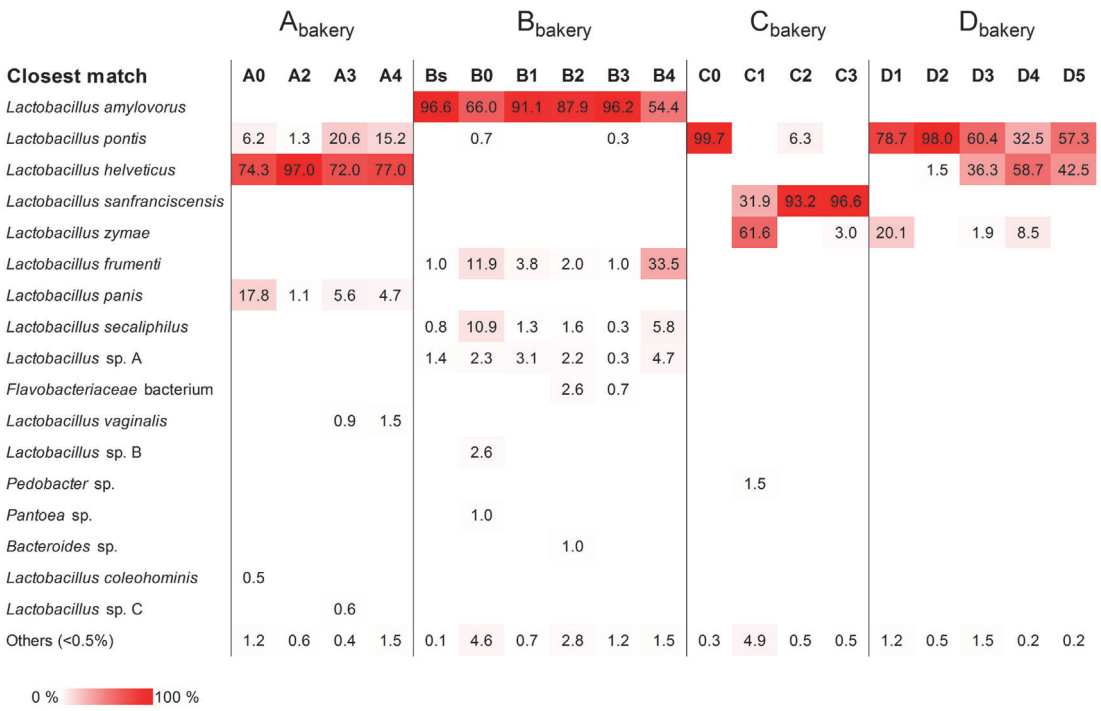

Fig 2. Relative abundance of bacterial species/genera detected in rye sourdoughs from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) using pyrosequencing of 16S rRNA gene amplicons. Samples are coded according to the description reported in Table 1.

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composition of the samples (Fig 4). Two-dimensional non-metric multidimensional scaling (NMDS) provided a stress value of 0.156 and an R2 value of 0.885. The stress value decreased to 0.064 and the R2 value increased to 0.977 when calculating the NMDS with three dimensions. Sourdough samples from A_{bakery} and B_{bakery} grouped in two different clusters, both characterized by closeness of the grouped samples, which illustrates the stability of both sourdough propagation processes. On the contrary, samples from both C_{bakery} and, especially, D_{bakery} grouped in looser clusters, thus indicating that the bacterial communities within these sourdoughs are less stable (Fig 4). 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 both contain *L. helveticus* and *L. pontis*. Samples collected at the B_{bakery} which employs a higher fermentation temperature, differ significantly from all other sourdoughs.

Discussion

This study evaluates the stability of both the microbial communities and chemical properties of rye sourdoughs from four Estonian bakeries. The bakeries used flour from the same source, but employed different propagation parameters. Our data showed that controlling the propagation conditions stabilized the cell density and distribution of prevalent LAB species in rye

Table 4. Number of reads, OTUs, expected OTUs at 500 reads and rate of new OTUs at 500 reads obtained from 16S rRNA pyrosequencing of rye sourdoughs from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}). Samples are coded according to the description reported in Table 1.

Sample	Raw reads	Reads	OTUs	Expected OTUs at 500 reads	Rate of new OTUs at 500 reads
A0	1929	1574	15	8.745	0.008
A1	NA*	NA	NA	NA	NA
A2	4332	4153	10	4.941	0.003
A3	2740	2335	12	6.750	0.004
A4	2320	1952	22	10.561	0.011
Bs	2417	2281	7	4.643	0.001
B0	1259	303	21	NA	NA
B1	2462	1260	12	7.414	0.007
B2	859	503	18	17.940	0.020
B3	932	688	14	11.589	0.014
B4	2179	1334	17	9.875	0.010
C0	2754	2749	7	2.389	0.003
C1	1410	263	15	NA	NA
C2	1960	1922	9	4.349	0.004
C3	3569	3437	12	4.149	0.004
D1	1415	1314	13	7.186	0.009
D2	2435	2356	7	3.661	0.002
D3	991	593	11	9.878	0.012
D4	3122	2923	10	4.197	0.002
D5	3303	2966	6	2.815	0.002

* NA–not acquired

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sourdoughs during long term propagation. The cell density of culturable LAB fluctuated in sourdoughs fermented at ambient temperature. High LAB cell densities correlate with high titratable acidity and low pH, which are both characteristic of mature rye sourdough and prerequisite for producing rye bread with desirable sensory properties. B_{bakery} sourdough samples showed the highest acidity because of the highest fermentation temperature. In contrast, samples collected in both C_{bakery} and D_{bakery} were characterized by insufficient acidity during the winter, probably due to the combination of low ambient temperature and short fermentation cycle. Adoption of a constant fermentation temperature (30°C) by D_{bakery} resulted in the stabilization of acid production and LAB cell density, even in this small scale bakery.

Higher stability of LAB communities was found in sourdough fermented at controlled conditions, compared to those fermented at ambient temperature that seemed strongly affected by the season of collection. Generally, the number of cycles of propagation of type II sourdoughs is lower than type I sourdough due to instability of the microbial community. The starter bacteria are often outcompeted by microorganisms contaminating flour and bakery environment. However, the sourdoughs collected in both large-scale bakeries (A_{bakery} and B_{bakery}) considered in this study showed better stability. This is probably due to the starter preparation chosen, which contained LAB communities that had been previously adapted to the propagation parameters employed in these bakeries. *L. helveticus* alone or *L. amylovorus* together with *L. frumenti* were the dominant LAB species in A_{bakery} and B_{bakery}, respectively. *L. amylovorus* and *L. frumenti* were previously identified as prevalent in other industrial rye sourdoughs propagated at elevated fermentation temperatures [4–5], similar to that (42–44°C) applied in B_{bakery},

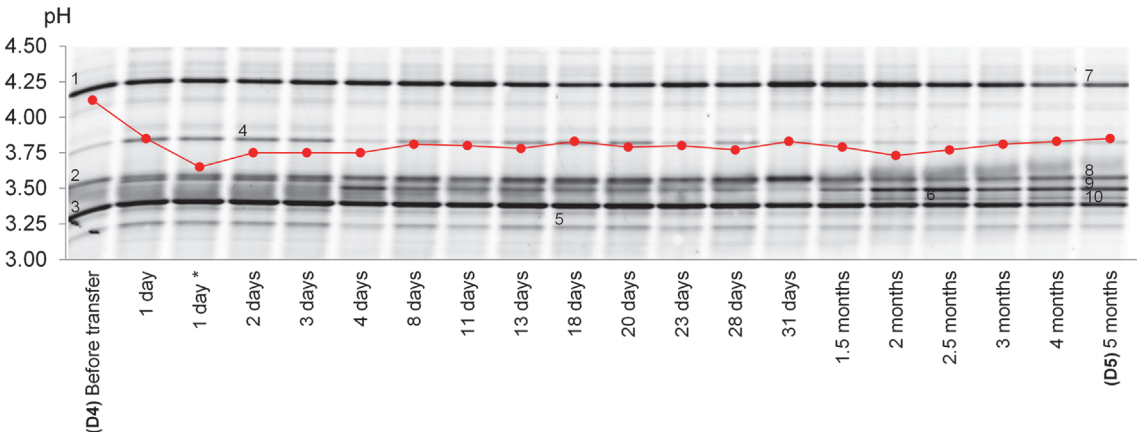

Fig 3. pH (red plot) and lactic acid bacterium species detected by DGGE analysis of the 16S rRNA gene amplicons in rye sourdoughs collected at the D_{bakery} before (D4) and after applying the new propagation protocol. Time after transfer is indicated below the gel (d–day; m–month). Bands: 1, 7 –*Lactobacillus 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.

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and are characterized by strong thermo- and acid-tolerance [27]. The persistence of *L. amylovorus* in sourdoughs may be also attributed to its high amylolytic activity and ability to produce bacteriocine amylovorin, a common feature for representatives of this species [28–29]. The same fingerprint type of dominant *L. amylovorus* was detected throughout the study, including the fresh sourdough (B0) from another bakery belonging to the same corporation. Adaptation to the sourdough environment could be the reason for such remarkable stability.

Although *L. helveticus* is not a common dominant species in sourdoughs [4, 13, 16] we found it as dominant bacterial species also in the sourdough of the small scale D_{bakery}. However, in contrast with A_{bakery}, the sourdough fermentation at ambient temperature in D_{bakery} prevented the stable prevalence of thermophilic *L. helveticus*. Indeed, depending on the season, *L. pontis* and *L. zymae* prevailed over *L. helveticus*. *L. zymae*, a species capable of growing at lower temperatures, has previously been found in both Greek and Belgian wheat sourdoughs, which indicates that it is widely spread [30–31]. New propagation parameters (higher fermentation temperature, decreased inoculum size, prolonged fermentation time, and use of 4°C refrigeration during breaks in production) adopted in D_{bakery} stabilized the LAB community and triggered an increase in the proportion of *L. helveticus* and *L. pontis*. No new species originating from raw materials or bakery environment were detected in the sourdough community of D_{bakery} even after five months of using the new protocol. This suggests high competitiveness and robustness of the dominant LAB that had adapted to different temperatures and initial sourdough acidity, although the house microbiota of the bakery may have also been the source of these LAB. The importance of house microbiota in the stability of sourdough microbial communities has been shown [12, 32].

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 applied in D_{bakery}. Unfortunately, comparing representatives of *L. pontis* with those contained in the commercial starter used in this bakery was not possible since no *L. pontis* was isolated. In contrast with the D_{bakery} sourdough, *L. sanfranciscensis* was also identified among prevailing bacteria in sourdough samples from C_{bakery}. *L. sanfranciscensis* is

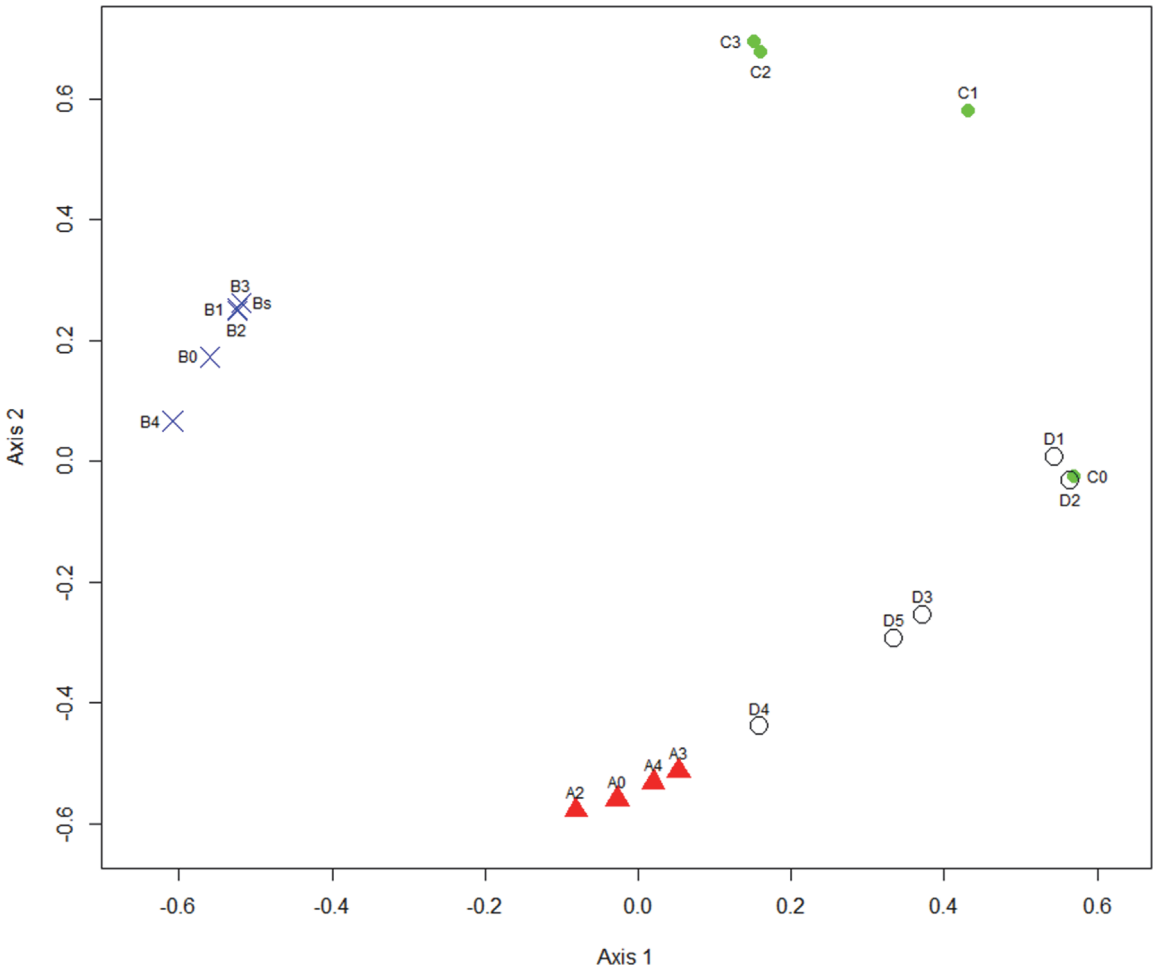

Fig 4. Two dimensional non-metric multidimensional scaling (NMDS) of sourdough samples from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}).

doi:10.1371/journal.pone.0148325.g004

frequently found in type I sourdoughs due to its adaptation to sourdough conditions, its small genome, and metabolism [4]. Stable non-competitive association of this maltose-positive LAB species with maltose-negative yeast *C. humilis* exists in traditional sourdoughs [33]. *L. sanfranciscensis* species is capable of hydrolyzing maltose by intracellular maltose phosphorylase activity and thereby accumulate glucose in the environment for *C. humilis* to utilize [34]. *C. humilis* was the only yeast species identified in the sourdoughs of both small-scale productions C_{bakery} and D_{bakery}.

Co-existence of *L. helveticus* with the yeast species *K. telluris* was found in the sourdough samples collected at the A_{bakery}. *K. telluris* (formerly *Saccharomyces telluris*, *Arxiozyma telluris*) is mainly known to cause infections in rodents and it may be isolated from soil [35].

Occurrence of this species in sourdough has not been previously reported. However, this thermophilic yeast is able to ferment glucose and grow on glucose, ethanol, and lactic acid [35]. As our identification is based only on culture-independent method (sequencing of 28S rRNA), further research should be carried out to assess the role of this yeast species in the sourdough community. The high fermentation temperature in B_{bakery} prevented the development of yeasts in the sourdough.

The culture independent methods applied in this study enabled us to identify LAB species (e.g. *L. secaliphilus* and *L. sanfranciscensis*) from sourdough and starter samples that were difficult to be cultivated. It has been previously shown that many sourdough LAB are sensitive to oxygen and/or have complex nutrient requirements [27, 36–37]. A wide variety of media should therefore be used to isolate sourdough LAB, since there is no universal medium that is suitable for all LAB. Culture independent methods such as DGGE and pyrosequencing 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 relative abundance of species within sourdough [16, 38].

Our data showed that sourdough bacterial communities within large-scale production facilities can be stable for many months using controlled propagation conditions, whereas, fermentation at room temperature leads to seasonal fluctuations in the species composition.

Supporting Information

S1 Fig. Lactic acid bacterium species detected by DGGE analysis of the 16S rRNA gene amplicons in rye sourdoughs from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}). Samples are coded according to the description reported in Table 1. Bands: 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*. Samples are coded according to the description reported in Table 1.
(TIF)

S2 Fig. Yeast species detected by DGGE analysis of the 28S rRNA gene amplicons in rye sourdoughs from three Estonian bakeries (A_{bakery}, C_{bakery} and D_{bakery}). Bands: 1, 2 –*Kazachstania telluris*; 3 –Cereal DNA; 4, 5, 6, 7 –*Candida humilis*. Samples are coded according to the description reported in Table 1.
(TIF)

Acknowledgments

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Author Contributions

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

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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

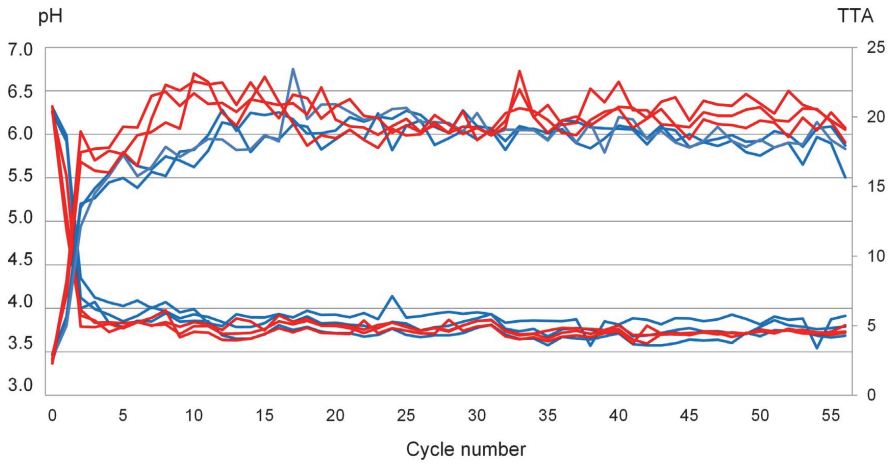

Figure 1. pH and total titratable acidity of six rye sourdoughs propagated at 20°C (20-I, 20-II and 20-III, shown in blue) and 30°C (30-I, 30-II and 30-III, shown in red) during 56 days.
doi:10.1371/journal.pone.0095449.g001

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).

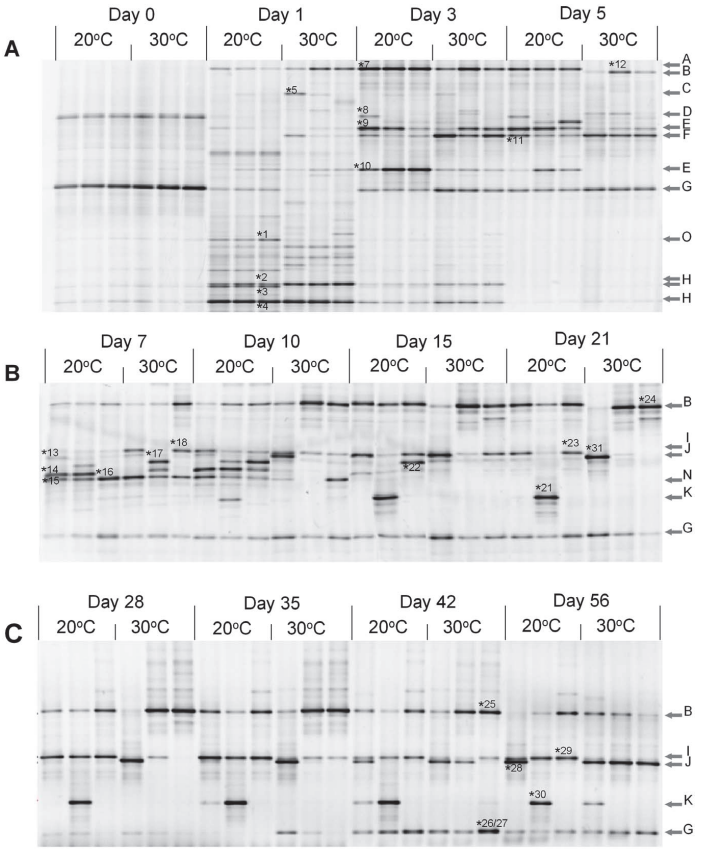

Figure 2. DGGE analysis of six rye sourdoughs propagated at 20°C (20-I, 20-II and 20-III) and 30°C (30-I, 30-II and 30-III) during 56 days. The arrows indicate specific bands for the following groups: (A) *Weissella* sp., (B) *Lactobacillus plantarum*, (C) *Enterobacteriaceae*, (D) *Lactobacillus* sp., (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

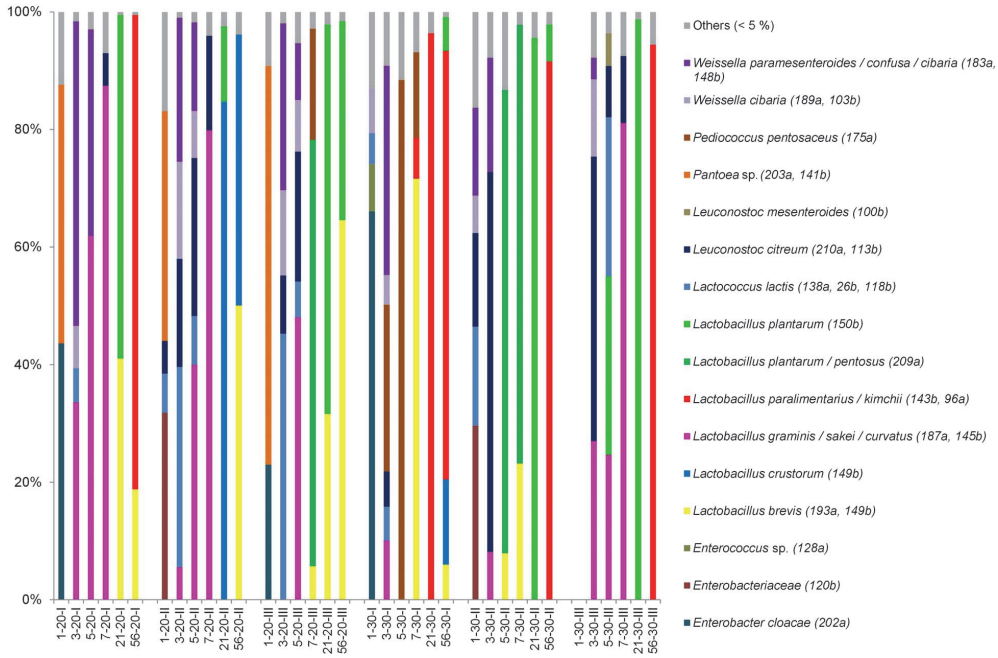

Figure 3. Pyrosequencing of 16S rRNA gene amplicons from spontaneous rye sourdoughs propagated for 56 days. Three sourdoughs were fermented at 20°C (20-I, 20-II, 20-III) and three sourdoughs fermented at 30°C (30-I, 30-II, 30-III). Sourdoughs were sampled at days 1, 3, 5, 7, 21, 56. The relative abundance 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

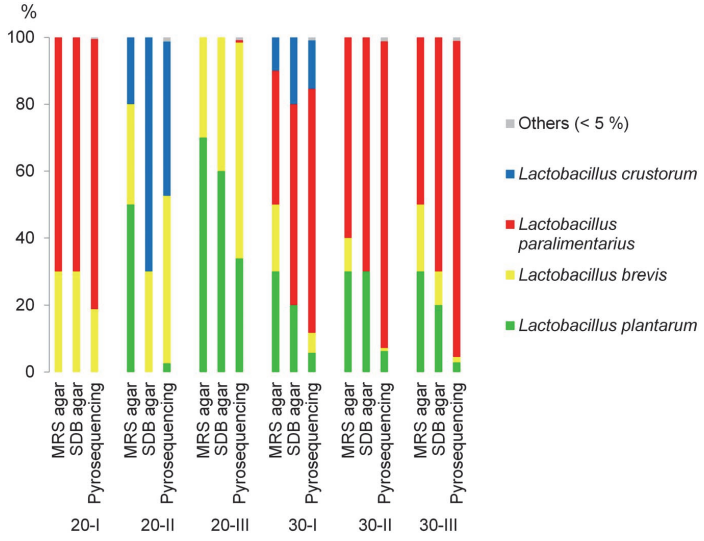

Figure 4. Ratio of species in the sourdoughs fermented at 20°C (20-I, 20-II, 20-III) or 30°C (30-I, 30-II, 30-III) after 56 backslopping cycles determined by plating on MRS and SDB media or by pyrosequencing of 16S rRNA gene amplicons.
doi:10.1371/journal.pone.0095449.g004

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 III

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Characterization of lactic acid bacteria isolated from spontaneously started rye sourdough and their potential application as starter bacteria for Type I sourdough production.

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Characterization of lactic acid bacteria isolated from spontaneously started rye sourdough and their potential application as starter culture for type I sourdough fermentation

M. Surženko[1,2], N. Part[1], E. Kelman[1], J. Rosend[1,2], K. Kontram[1,2], I. Sarand[2]*

[1]Center of Food and Fermentation Technologies, Tallinn, Estonia

[2]Department of Chemistry and Biotechnology, Tallinn University of Technology, Tallinn, Estonia

*Inga sarand

E – mail: inga.sarand@ttu.ee

Abstract

Four lactic acid bacteria strains, *Lactobacillus plantarum* M30I–1, *Lactobacillus brevis* M30I–2, *Lactobacillus paralimentarius* M30I–3, and *Lactobacillus cructorum* M30I–9, isolated previously from spontaneously started laboratory–scale rye sourdough, were examined as potential starter cultures for a type I sourdough process. We evaluated the individual growth of selected isolates in semi–liquid sourdoughs at two different temperatures using a TAMIII microcalorimeter. We also studied other technologically important parameters, such as acidification rate, aroma profiles, microbial counts, and their ability to co–exist with maltose–negative yeasts. Finally, we examined potential anti–microbial properties of selected strains and their ability to prolong the mould–free shelf–life of rye breads. We found that *Lb. brevis* M30I–2 has high potential as a starter culture for traditional rye sourdough. In addition to rapid growth and fast acidification of the dough at ambient temperature, this strain displayed both antibacterial activity and an ability to suppress the germination of bread spoilage fungi.

Introduction

Sourdough can be defined as a mixture of flour and water fermented with a microbial consortia of lactic acid bacteria (LAB) and yeasts (De Vuyst et al., 2009; De Vuyst and Neysens, 2005). Fermentation of the dough is essential for the production of rye bread due to the lack of gluten and the presence of active α–amylase in rye flour (Stolz, 2003; Corsetti and Settanni, 2007). Organic acids produced by LAB during fermentation improve the rheological properties of the dough and decrease the inactivation temperature of undesirable amylolytic enzymes while yeasts emit carbon dioxide, thus increasing loaf volume (Stolz, 2003, De Vuyst and Neysens, 2005). In addition, both sourdough LAB and yeasts produce a whole range of flavor compounds

that contribute to the formation of the overall sensory profile of rye bread (Damiani et al., 1996; Kirchhoff and Schieberle, 2001). Metabolites produced by LAB during fermentation also play a crucial role in the bioprotection of baked products against both bacterial and fungal spoilage (Schnürer and Magnusson, 2005; Gerez et. Al., 2009; Dalie et al., 2010). In a modern world of "green" products, where the use of chemical preservatives is restricted by food legislation and often rejected by costumers, bio preservatives produced by sourdough microflora could become crucial for the survival of artisanal bakeries (Leroy and De Vuyst, 2004; Dalie et al. 2010; Hassan et al. 2015).

Sourdoughs can be divided into three types depending on fermentation conditions applied (Böcker et al., 1995; De Vuyst and Neysens, 2005). Type II sourdoughs are characterized by high dough yield and elevated fermentation temperatures (> 30 °C) and are typically used within facilities that have a high production rate. In contrast, small–scale bakeries use the so–called traditional (Type I) sourdough production technology determined by ambient propagation temperatures and firm or semi–liquid consistency (De Vuyst and Neysens, 2005). In contrast with Type II sourdoughs, whose microbial diversity is restricted by low pH and high fermentation temperatures, traditional sourdoughs may be subjected to season–dependent fluctuations in microbial composition due to unstable propagation conditions (Viiard et al., 2016).

The typical bacterial consortia of Type I sourdough mainly consists of obligately and facultative heterofermentative LAB while fungi are often represented by maltose negative yeasts (Gänzle et al., 1998; De Vuyst and Neysens 2005; De Vuyst et al., 2009). Because the use of baker's yeasts in some small–scale bakeries is restricted by the technological scheme applied, the number of indigenous yeast in sourdough should be high enough to produce a sufficient amount

of carbon dioxide. It has been reported that the bacterial and fungal populations within Type I sourdough often interact with each other through shared pools of metabolites and nutrients. Consequently, changes in the composition of dominant bacteria species in sourdough can switch mutualistic associations between LAB and yeasts to interspecific competition (De Vuyst et al., 2009). This, in turn, can influence the quality and volume of the baked product either by decreasing the gas holding capacity due to the insufficient acidification of the dough in case of decreased LAB counts, or by diminishing the amount of carbon dioxide emitted as a result of the bacteria outcompeting the yeasts (Martinez–Anaya, 2003).

For artisanal and small–scale industrial bakeries that still adopt a Type I sourdough fermentation process, the use of starter bacteria that are highly adapted to unstable fermentation conditions could be a rational way of maintaining the quality of the rye breads they produce. Selection of starter bacteria for industrial purposes often relies only on some a specific technological parameter such as fast acidification while ignoring other critical aspects like bioactivity in different types of sourdoughs and robustness in the sourdough cycle under unstable propagation conditions (Leroy and De Vuyst, 2004). As a result, starter strains are often outcompeted in a long–term propagation process by undesirable microflora which often induces fermentation failures and unstable product quality (De Vuyst et al., 2009).

In the present study we implemented a complex approach to select bioactive, competitive, and functional starter bacteria for a traditional sourdough process. Potential starter LAB – obligate heterofermentative strain (*Lactobacillus brevis* M30I–2) and facultative heterofermentative strains (*Lactobacillus plantarum* M30I–1 and *Lactobacillus paralimentarius* M30I–3 and *Lactobacillus crustorum* M30I–9) were isolated from spontaneously started Type I rye sourdough (Bessmeltseva et al. 2014). These strains dominated in sourdoughs after two

88 month of propagation at ambient temperatures (20 and 30°C), so their metabolism should be well
89 adapted to the Type I rye sourdough ecosystem.

90 Materials and methods

91 Microorganisms used in this study

92 All LAB strains and fungi, with the exception of *Aspergillius brasiliensis* ATCC 16404,
93 were obtained from the CCFFT microbial collections (Tables 1 and 2). Reference
94 microorganisms were kindly provided by the Laboratory of Food and Environmental
95 Investigations (Institute of Food Safety, Animal Health and Environment – "BIOR", Latvia). The
96 cultivation of all microorganisms was carried out under the conditions listed in Tables 1 and 2.

97 Evaluation of the growth of single starter bacteria in solid 98 sourdoughs using a TAM III Microcalorimeter

99 Starter LAB were cultivated as described in Table 1. Four dough batches were prepared
100 from 10 KGy irradiated rye flour and a sterile 0.5% NaCl solution (1:1). Each batch was
101 inoculated with a single culture of starter LAB to achieve a final concentration of 10^6 colony
102 forming units (CFU) g^{-1} bacterial cells in the dough. The batch inoculated by *Lb. plantarum*
103 M30I–1 is referred to as "A", while batches inoculated by *Lb. brevis* M30I–2, *Lb.*
104 *paralimentarius* M30I–3 and *Lb. crustorum* M30I–9 were referred to as "B", "C" and "D"
105 respectively. After mixing for 15 minutes at 100 rpm in a Stomacher 400 circulator (Seward
106 Limited, England) each dough was incubated in the thermostat at 20 or 30°C. After 12h of
107 incubation all sourdoughs were refreshed 1:10 by mixing with a new portion of sterile flour and
108 0.5% NaCl solution. After mixing, a 1g aliquot of each experimental dough was transferred into

a 3 ml microcalorimetric vial. The vials were then incubated in the Thermal Activity Monitor (TAM) III microcalorimeter (TA Instruments, New Castle, DE, USA) at 20°C (A20, B20, C20, D20) or 30°C (A30, B30, C30, D30) for 12h. The remaining dough samples were incubated in stomacher bags at 20°C or 30°C in parallel with the calorimetry analysis.

The power–time curves of potential starter LAB were measured in a TAM III microcalorimeter with 5 minutes intervals. To evaluate the matrix effect on the heat flow, a "blank" dough was prepared by mixing 10 KGy irradiated rye flour with sterile 0.5% NaCl solution (1:1) without adding bacterial culture.

The "blank" heat flow signal was treated as a background signal of the dough matrix and substracted from all other microcalorimetric power-time curves. Each curve was then analyzed as described by Kabanova et al (2012). After the 12h fermentation cycle we determined the concentrations of both lactic and acetic acid as well as Total Titratable Acidity (TTA) and bacterial counts in all samples. The pH of each dough was measured every three hours during the 12h fermentation cycle.

Analysis of pH and TTA

Five grams of fermented sourdough was homogenized with 45 ml of distilled water using a Polytron PT2100 homogenizer (Kinematica AG, Switzerland). Both the pH and TTA were measured using a Food and Beverage analyzer D22 (Mettler–Tolledo International Inc., USA). Each analysis was performed in two parallels.

HPLC Analysis of lactic and acetic acid

We measured the concentrations of both lactic and acetic acids in each mature sourdough using High Performance Liquid Chromatography (HPLC). Our HPLC setup consisted of a

separations Module Waters 2695 Alliance used together with a Refractive Index Detector 2414 (both from Waters Corporation, USA). Samples were prepared by homogenizing 1g of fermented dough with 9ml of MilliQ water. Samples were then filtered through a 45µm Whatman syringe nylon filter (25mm GD/X) and injected into an HPLC Organic Acid Analysis column (Aminex HPX–87H ion exclusion column, 300mm X 7.8mm (Bio–Rad Laboratories Inc., USA). A sulfuric acid solution (2.2 ml per 1 L MilliQ water) was used as a solvent with a flow rate 0.6 ml min^{-1} at 35°C. All analysis were performed in triplicate.

Extraction and identification of the volatiles mature doughs

The extraction of dough volatiles was carried out using solid–phase microextraction (SPME). SPME was conducted by exposure to the headspace of the sample (HS–SPME). 1g of each mature sourdough sample was placed into a 20 ml glass autosampler vial capped with a PTFE/silicone septum. 7.5 µg L−1 of 2–chrolo–6–methylphenol was added into each vial as an internal standard. Vials were pre–incubated at 45 °C for 5 minutes. An SPME fiber (30/50µm DVB/Car/PDMS Stableflex, length 2 cm; Supelco, Bellefonte, PA, USA) was then used to extract the volatile compounds from the headspace for 20 minutes while stirring at 45°C until they reached equilibrium.

Identification and quantification of dough volatiles were performed using a Micromass GCT Premier gas chromatograph system (Waters, Milford, MA, USA) coupled with a PAL autosampler (CTC Analytics AG, Lake Elmo, MN, USA). After SPME procedure, the volatile compounds were desorbed in splitless mode into a GC injection port equipped with a 0.75 mm internal diameter liner at 250°C for 10 minutes. A DB5–MS column (30 m length × 0.25 mm i.d. × 1.0 µm film thickness; J&W Scientific, Folsom, CA, USA) was used with helium as a carrier gas at a flow rate of 1.0 ml min−1.

The oven was programmed to ramp up from 45°C at a rate of 10°C min−1 to a final temperature of 280°C with an additional holding time of one minute (total run time 24.50 min). Two analytical replicates were used for each sourdough sample. Identification and quantification of volatile compounds were carried out using MassLynx software (Waters, Milford, MA, USA).

Studying the co–cultivation of single starter culture with maltose–negative yeasts

Starter LAB and *Candida humilis* SR1 were pre–cultivated as described in Tables 1 and 2. Doughs prepared as described in microcalorimetry section were inoculated with a single culture of selected starter LAB and *C. humilis* SR1 strain to achieve a final concentration of 10^6 CFUg-1 bacterial cells and 10^4 CFUg-1 yeast cells in the dough. After mixing, each sourdough was divided in 2 portions. One portion was incubated in the stomacher bags for 12h at 20 °C and another at 30 °C. After 12h of incubation, the sourdoughs were refreshed 1:10 by mixing with a new portion of sterile flour and water. In total, each experimental sourdough went through fourteen refreshment cycles. The TTA, pH, and microbial counts in the experimental doughs were measured at the end of the 14^{th} fermentation cycle.

Determination of microbial counts in fermented doughs

Five grams of fermented sourdough was homogenized with 45ml of sterile 0.5% NaCl solution and a line of decimal dilutions was performed. To evaluate bacterial counts, 50µl from a suitable dilution was plated onto SDB agar containing 100 µg l-1 cycloheximide (Sigma–Aldrich, USA). Yeast counts were measured by plating of 50µl from a suitable dilution onto YPD agar plates containing 100µg l-1 chloramphenicol (Sigma–Aldrich, USA). Petri dishes

were incubated overnight at 30 °C under aerobic conditions. CFU were counted from agar plates with suitable dilutions.

In vitro anti–microbial assays

Potential starter LAB were tested for their ability to suppress the growth of reference microorganisms using an agar well diffusion assay and a dual culture overlay assay as earlier described by Magnusson and Schnürer (2001) with some modifications: for the agar–well diffusion assay, LAB and reference bacteria strains were cultivated in the liquid medium described in Table 1. Bacterial cells from the LAB suspension were removed by centrifugation for 10 minutes at 11000rpm min-1 followed by filtration through a 0.2µm filter. The supernatant was then divided into two parts. One part was neutralized by titration using a 30% NaOH solution. For the reference bacteria, concentrations of bacterial cells were determined using a hemocytometer and adjusted to 10^6 CFU ml-1 using sterile 0.5% NaCl solution. Reference mould was cultivated as described in Table 2. Spores were collected from the plate surface by shaking them with 1.5 ml of sterile 0.5% NaCl solution. The spore concentration was determined using a hemocytometer and adjusted to 10^6 spores ml-1.

50µl of either reference bacteria or spore suspension was spread plated onto agar plates that contained the suitable cultivation media (Sabouraud dextrose agar for mould cultivation or Nutrient agar for bacteria cultivation). After that, three wells (5mm in diameter) were made in the agar surface using a sterile cork borer. The first well was filled with 50µl of cell–free supernatant, the second with 50µl of neutralized supernatant and the third was left empty as a negative control. Plates were then left for 3 hours at room temperature and then placed inside incubators with suitable temperatures (25 °C for the anti–mould assay and 37°C for the anti–

bacterial assay). Plates were incubated up to 7 days. Both the presence and size of inhibition zones around each well was checked daily.

A dual culture overlay assay was made as follows: pure cultures of LAB were cultivated overnight in SDB broth at 30°C. Reference bacteria and moulds were pre–cultivated as described above. Five µl of LAB pure cultures were spot–inoculated onto the middle of SDB agar plates. The inoculated plates were incubated for 48 h at 30°C and then overlaid with 20 ml Sabouraud agar contaminated with 10^6 spores of reference mould or 20 ml Nutritional agar inoculated with 10^6 bacterial cells. After solidification the agar plates contaminated with mould were incubated at 25°C for 7 days and plates inoculated with reference bacteria were incubated at 37°C for 72h. Both the presence and size of inhibition zones around the LAB spots was checked daily. All antimicrobial assays were made in triplicate.

In situ anti–fungal assay

Each experimental sourdough was prepared as described above. The first batch was inoculated with *Lactobacillus brevis* M30I–2 pure culture to obtain a final concentration 10^6 CFU g-1. The second batch, inoculated with *Lactobacillus crustorum* M30I–9 pure culture was used as a negative control. Sourdoughs were then fermented at 30°C for 12h. The resulting sourdoughs were used to preparae rye dough according to the following recipe: sourdough – 33%, sterile water – 28%, sterile flour – 39%. The doughs were then fermented for 110 minutes at 30°C, put into baking forms and proofed at 30°C for an additional 45 minutes. Baking was carried out in a Self Cooking Centre (Metos Systems Rational, Weikersheim, Germany) as follows: 10 minutes at 230°C, 15 minutes at 200°C, 15 minutes at 180°C, and 10 minutes at 150°C. The Baked breads were cooled to room temperature, sliced, and placed onto petri dishes. Both the cooling and slicing procedures were made under sterile conditions. Reference moulds

were pre–cultivated as described in Table 1. Spores were collected from the plate surface by shaking them with 1.5 ml of sterile 0.5% NaCl solution. The mould spore concentration was determined using a hemocytometer and adjusted to 10^6 spores ml-1. Bread slices were spot inoculated using 5µl of spore suspension or by a piece of reference mould mycelium 5 mm in diameter and stored at 25°C for a week. Visual signs of mould germination were checked daily.

Results

Growth and bioactivity of a single starter culture in semi–solid sourdoughs propagated at 20 and 30°C

Power–time curves obtained after the cultivation of *Lb. plantarum* M30I–1, *Lb. paralimentarius* M30I–3 and *Lb. crustorum* M30I–9 strains at 30°C in rye sourdough demonstrated high similarity in both maximum heat flows (P_{max}) and duration of exponential growth phase (T_{Pmax}). In contrast, the power–time curve of *Lb. brevis* M30I–2 cultivated at 30°C had significantly higher P_{max} at the latest stage of the fermentation (Fig 1, Table 3). The maximum specific growth rates achieved by *Lb. plantarum* M30I–1, *Lb. brevis* M30I–2, *Lb. paralimentarius* M30I–3 and *Lb. crustorum* M30I–9 strains were 0.472, 0.354, 0.392 and 0.451 h^{-1} respectively (Table 3). All starter LAB were able to finish exponential growth phase in three hours after the inoculation and reach high biomass density (10^9 $CFUg^{-1}$) at the end of fermentation cycle (Table 3). The pH values of all tested doughs were quite similar and lied in the range 3.62–3.93 at the end of the fermentation. However, dough inoculated by *Lb. brevis* M30I–2 strain had significantly higher TTA values comparing to other doughs. The HPLC analysis of mature sourdoughs revealed up to four times higher amounts of acetic acid in the

241 batch inoculated with *Lb. brevis* M30I–2 comparing with batches inoculated with the other LAB
242 studied (Table 3).

243 When the bacteria were cultivated at 20°C, the maximum specific growth rates of *Lb.*
244 *plantarum* M30I–1*, Lb. paralimentarius* M30I–3 and *Lb. crustorum* M30I–9 decreased up to 4
245 times comparing to those achieved during the cultivation at 30°C. *Lb. brevis* M30I–2 was the
246 only strain whose maximum specific growth rate decreased only two times (Table 3). The lower
247 fermentation temperature had a negative effect on the acidifying activity of the bacteria tested.
248 Thus, the amount of lactic acid in samples fermented with *Lb. paralimentarius* M30I–3 and *Lb.*
249 *crustorum* M30I–9 at 20°C were three to five times lower comparing to samples fermented with
250 the same strains at 30°C (Table 3). However, the production of lactic acid by *Lb. plantarum*
251 M30I–1 and *Lb. brevis* M30I–2 strains was less affected by the lower fermentation temperature
252 (Table 3).

253 Characterization of volatile compounds in sourdoughs inoculated
254 with single starter culture.

255 Six alcohols (Ethanol, 2,3–Butanediol, 2–Ethyl–1–hexanol, 1–Octanol, 1–Dodecanol and
256 2–Methyl–1–butanol) were found in sourdoughs fermented using the LAB strains under study.
257 Most were found in all sourdoughs tested. One exception was 2–Methyl–1–butanol which was
258 not detected in samples inoculated by *Lb. brevis* M30I – 2 strain (Table 4).

259 Esters, detected in all studied sourdoughs were represented by Ethyl acetate, Isoamyl
260 acetate, Hexyl acetate, Phenylethyl acetate, and Ethyl undecanoate. Propyl decanoate was found
261 in all sourdoughs except those inoculated by *Lb. brevis* M30I–2 strain (Table 4).

262 The most considerable difference in the composition of the volatile compounds within the
263 strains we tested was found between the aldehyde components. Five different aldehydes were

detected in the fermented doughs and only Butyrolactone was found in all samples. Sourdoughs fermented by *Lb. brevis* M30I – 2 demonstrated the most unique aroma profile which consisted of Butyrolactone, (z)–2–Decenal, and (E,E)–2,4–Decadienal (Table 4). The last two compounds were only found in doughs inoculated with *Lb. brevis* M30I – 2 strain. Three other strains demonstrated a similar set of aldehyde fractions and consisted of Butyrolactone, Benzaldehyde and (E,Z)–2,4–Decadienal (Table 4).

Seven organic acids: dodecanoic, nonanoic, octanoic, heptanoic, 2–Ethylhexanoic, butyric and acetic acids were found in all doughs studied (Table 4). A butyric acid related peak intensity was especially high in the sourdoughs fermented using *Lb. brevis* M30I–2. This sample, fermented at 30°C, demonstrated a nine fold increase in the corresponding peak intensity (data not shown).

Influence of single starter bacteria on yeast counts during fourteen cycles of co–cultivation in Type I sourdough

Sourdoughs started with a single starter bacteria in combination with yeast *Candida humilis* were analysed after the 14^{th} propagation cycle. The results demonstrated high similarity in pH values and bacterial counts irrespective of the fermentation temperature applied (Table 5). The TTA values of the doughs inoculated with *Lb. plantarum* M30I–1, *Lb. paralomentarius* M30I–3 or *Lb. crustorum* M30I–9 were similar and lied in the range 15.07–15.67 for doughs fermented at 20°C and 17.32–18.47 for those, fermented at 30°C. Doughs fermented by *Lb. brevis* M30I–2 demonstrated higher TTA values at both temperatures. This is probably due to the higher amount of acetic acid, which is present in mature dough mostly in an undissociated form (Table 5). Yeast counts in the sourdoughs studied varied depending on the fermentation temperature applied. In sourdoughs fermented by *Lb. paralimentarius* M30I–3 and *Lb.*

crustorum M30I–9, yeast counts were 10^8 and 10^7 $CFUg^{-1}$ for doughs fermented at 20°C and 30°C, respectively. The highest difference between yeast counts was found in doughs fermented at selected temperatures in sourdoughs inoculated with *Lb. plantarum* M30I–1 where fermentation at 30°C resulted in the lowest count of yeast cells (10^6 $CFUg^{-1}$) within all doughs tested. Finally, the same number of yeast cells (10^7 $CFUg^{-1}$) was detected in doughs fermented at both temperatures when *Lb. brevis* M30I–2 was used as the starter culture.

Influence of single starter culture on the growth of potential pathogens and reference mould *in vitro*

In the present study, each potential starter bacteria was screened for their ability to suppress the growth of food–borne pathogens: *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 10876, *Yersinia enterocolitica* ATCC27729 and *Staphylococcus aureus* ATCC25923. All LAB tested showed inhibiting activity of moderate to high intensity against all tested foodborne pathogens when tested in the form of active biomass (Table 6). Conversely, cell–free supernatants (CFS) obtained from each LAB strain demonstrated only limited inhibiting activity. Both CFS from *Lb. plantarum* M30I–1 and *Lb. paralimentarius* M30I–3 strains were able to inhibit the growth of *B. cereus* ATCC 10876 and *S. aureus* ATCC25923 to a minor degree and demonstrate moderate inhibition of the growth of *E. coli* ATCC 25922 and *Y. enterocolitica* ATCC27729. CFS from *Lb. brevis* M30I–2 showed weak inhibition of *E. coli* ATCC 25922 and *Y. enterocolitica* ATCC27729, while moderate inhibiting activity was detected against *B. cereus* ATCC 10876. Moderate inhibition of *E. coli* ATCC 25922 and *Y. enterocolitica* ATCC27729 together with weak inhibition of *B. cereus* ATCC 10876 was detected in CFS from *Lb. crustorum* M30I–9. Finally, CFS obtained from both *Lb. brevis* M30I–2 and *Lb. crustorum* M30I–9 strains did not inhibit the growth of *S. aureus* ATCC25923 (Table 6).

310 *In vitro* anti–fungal activities of starter LAB were tested using *Aspergillus brasiliensis*
311 *ATCC 16404* as reference fungi. As a result, among all LAB tested, only *Lb. brevis* M30I–2 was
312 able to suppress the germination of the reference mould. We detected strong inhibition of mould
313 growth when *Lb. brevis* M30I–2 was tested in the form of an active biomass and weak inhibition
314 was observed in trials with CFS (Table 6).

315 The effect of *Lb. brevis* M30I–2 on the germination of seven mould
316 strains isolated from spoiled rye breads

317 The anti–fungal activity of *Lb. brevis* M30I–2 was further verified in an *in situ* study.
318 Prepared breads were contaminated by four *Aspergilli* and two *Penicillium* species. In control
319 samples prepared using *Lb. crustorum* M30I–9 as a starter culture the first sings of mould
320 germination appeared already after the first day of storage (data not shown). In contrast, the shelf
321 life of breads prepared using *Lb. brevis* M30I–2 as a starter culture were prolonged up to seven
322 days except for breads contaminated by *Penicillium corylophilum* A111. The spores of the latter
323 germinated already in the fourth day of storage (Table 7).

324 Discussion

325 Stable and metabolically active microbial consortia are essential for the production of
326 high–quality rye breads (Stolz 2003; De Vuyst and Neysens 2004; De Vuyst et al., 2009).
327 Because fermentation temperature is one of the key factors that affect microorganisms in the
328 sourdough cycle (Viiard et al., 2016; De Vuyst et al., 2014; Minervini et al., 2014 Vrancken et
329 al., 2011; Neysens and De Vuyst 2005; Brandt et al., 2004), bakeries that propagate sourdough at
330 ambient temperature require starter cultures that have the ability to grow and acidify the dough
331 over a wide temperature range. The fermentation temperatures and strains used in our trials

resulted in significantly different metabolic performance. When starter LAB were fermented at 30°C, all strains demonstrated rapid growth with a shorter exponential growth phases and higher maximum heat flow compared with doughs fermented at 20°C. Starter LAB also displayed high acidifying activity and successfully dropped the pH below 4.3 after the 6th hour of fermentation at 30°C. It is widely accepted that higher fermentation temperatures favor the acidification process because of the increased production of lactic acid (De Vuyst et al., 2014). Indeed, the concentration of lactic acid found in samples fermented at 30°C were up to four times higher compared with sourdoughs fermented at 20°C, however, we observed only slight changes in the amount of acetic acid.

Cultivation at 20°C drastically influenced the performance of potential starter bacteria. The maximum specific growth rates of *Lb. plantarum* M30I–1, *Lb. paralimentarius* M30I–3 and *Lb. crustorum* M30I–9 strains decreased up to four times compared with the rates achieved during cultivation at 30°C. *Lb. brevis* M30I–2 was the only strain whose maximum specific growth rate was significantly less affected by a decrease in cultivation temperature. The lower fermentation temperature had a negative effect on the acidifying activity of all bacteria we tested. Thus, only *Lb. plantarum* M30I–1 and *Lb. brevis* M30I–2 were active enough to drop the pH of the fermented doughs below 4.3 by the end of the fermentation cycle. This pH is considered a benchmark of a successfully developed fermentation process (Corsetti, 2013). It has been shown that microorganisms that grow in the transition between the exponential and stationary growth phases are preferable for sourdough propagation due to their higher cell counts and acidifying activity (Gaggiano et al. 2006). Among all LAB strains tested, only *Lb. brevis* M30I–2 was able to end exponential growth phase within 12h when inoculated within doughs fermented at 20°C. The high activity of *Lb. brevis* M30I–2 at 20°C could explain the predominance of this strain in

355 the microbial population of spontaneously started Type I rye sourdoughs propagated during two
356 months at 20°C (Bessmeltseva et al., 2014).

357 Organoleptic properties, especially taste and aroma are important factors that define
358 customer preferences in rye breads (Salim–ur–Rehman et. Al., 2006). In bread making, both the
359 fermentation and baking processes affect the final aroma of product (Hui et. Al., 2006). It is
360 considered that baking process defines final aroma of the crust with its typical roasty notes,
361 whereas the sensory properties of the crumb are developed mainly due to the activity of
362 microorganisms during dough fermentation (Corsetti and Settanni. 2007). Moreover, it has been
363 shown that sourdough breads have a wider aroma profile compared to chemically acidified
364 breads (Gobbetti and Gänzle, 2013). Due to their importance in the baking industry, one ought to
365 take into account the repeatability of achieving similar aroma profiles under unstable propagation
366 conditions and select starter bacteria that are robust to changing conditions. All strains tested in
367 this study demonstrated stable qualitative composition of volatiles at both propagation
368 temperatures tested. Sourdoughs inoculated with *Lb. plantarum* M30I–1, *Lb. paralimentarius*
369 M30I–3 and *Lb. crustorum* M30I–9 showed identical composition of volatile compounds,
370 whereas a unique profile of aldehyde compounds was found in doughs fermented with *Lb. brevis*
371 M30I–2. Aldehydes are assumed to be the one of the key flavor compounds in rye based baked
372 products and (E,E)–2,4–Decadienal has been previously reported as a contributor to the overall
373 flavor of the crumb in rye breads (Burdock, G. A. 2009; Kirchhoff and Schieberle, 2001).

374 A few of the alcohols we identified in this study have previously been found in traditional
375 sourdoughs from different origins. 1–Octanol was detected in a Type I wheat sourdough
376 fermented with *Lb. brevis* (Damiani et al., 1996), and ethanol was previously described as a
377 volatile compound in both a Type I wheat sourdough (Damiani et al., 1996, Di Cagno et al.,

2014) and Type I rye sourdough (Kaseleht et al., 2011). Finaly, 2–Methyl–1–butanol was identified in a traditional wheat sourdough that originated from southern Italy (Di Cagno et al., 2014). The latter, together with 2,3–Butanediol, was detected in our sourdough samples, although they are usually associated with yeast fermentation. However, 2,3–Butanediol could be also produced by LAB from pyruvate via diacetyl or acetoin (Pasteris and De Saad, 2005). This compound provides a characteristic "creamy" or "buttery" odor. The interactions of acids and alcohols are assumed to be the predominant way of ester formation in sourdoughs (Burdock, G. A. 2009). Because ethanol is considered to be the main alcohol in the sourdough matrix, one could assume the predominance of ethyl forms of esters in fermented doughs (Burdock, G. A. 2009). Indeed, the intensity of Phenylethyl acetate and Ethyl acetate peaks was up to ten times higher comparing to the peaks of other ester compounds (data not shown). Of all the compounds identified, only Ethyl acetate was frequently reported as a volatile compound found within traditional sourdoughs (Damiani et al., 2009; Kaseleht et al., 2011; Di Cagno et al., 2014). However, another ester identified in this study, Hexyl acetate, was also previously detected in rye sourdough by Kaseleht et al. (2011).

Besides technologically important parameters such as dough acidification and volatile formation, selection of starters for Type I sourdoughs should also take into account rapidly growing customer demand in baker's yeast–free rye breads. (Stolz, 2003). Studying associations between potentials starter bacteria and endogenous yeast species within Type I sourdoughs is essential for the production of a starter that can fulfill both the needs of the end customer and baker. In high quality mature sourdough, the optimal ratio between yeasts and LAB considered to be about 1:100 (Gobetti et al., 1994; Ottogalli et al., 1996). In the sourdoughs we studied fermented with *Lb. brevis* M30I–2 this ratio remained constant at both temperatures we tested. In

401 the case of *Lb. plantarum* M30I–1, *Lb. paralimentarius* M30I–3, and *Lb. crustorum* M30I–9 a
402 decrease in fermentation temperature caused an increase in the yeast count in the sourdoughs
403 while the LAB counts remained the same. In a sourdough ecosystem lactobacilli could suppress
404 the growth of yeast both by outcompeting them for critical nutrients or by producing antifungal
405 compounds (Gobbetti and Gänzle2013). However, both high yeast and LAB counts in all
406 experimental sourdoughs we studied support a more mutualistic type of interaction between
407 these microorganisms.

408 Microbial–related spoilage of bread is considered to be one of the main factors that affect
409 the shelf–life of this type of baked product (Smith et al., 2004). Despite the fact that both a
410 relatively high acidity and low water activity of rye bread restrict the growth of contaminative
411 bacteria, some cases of bacteria–related foodborne outbreaks caused by baked products have
412 been reported (Smith and Simpson 1995). In this study, we screened potential starter bacteria for
413 their ability to suppress the growth of the food–borne pathogens *Escherichia coli* ATCC 25922,
414 *Bacillus cereus* ATCC 10876, *Yersinia enterocolitica* ATCC27729, and *Staphylococcus aureus*
415 ATCC25923. All LAB we tested showed inhibiting activity of moderate to high intensity against
416 all four foodborne pathogens when tested as an active biomass, however, they display only
417 limited inhibiting activity when cell–free supernatants (CFS) were tested. Because no
418 antibacterial activity was detected in neutralized CFS (data not shown), we can assume that the
419 inhibitory effect is a result of the acidic nature of the inhibitive compounds produced by the LAB
420 strains studied.

421 Unlike the relatively rare cases of bacterial contamination of sourdough breads, fungal
422 infections are reported to be a major cause of microbiological spoilage of rye bread (Legan,
423 1993; Smith et al., 2004). The modern baker has a whole list of chemical preservative agents at

their disposal (Brul and Coote, 1999; Davidson 1999). However, the addition of chemicals to food products is restricted by food legislation (Brul and Coote, 1999; Keshri et al., 2002) and often rejected by consumers (Smith et al., 2004). A number of studies have confirmed that certain LAB strains have antifungal activity (Rouse et al., 2008; Gerez et al., 2009, Ryan et al., 2011), including bacteria isolated from sourdough ecosystems (Dal Bello et al., 2007; Hassan et al., 2015). Thus, the use of starter bacteria that possess antimicrobial activities could become a natural alternative to chemical preservatives. In this study only *Lb. brevis* M30I–2 displayed significant antifungal activity in an *in vitro* assay. However, this effect completely disappeared after the CFS was neutralized (data not shown). These results suggest that the observed antifungal activity of the strain was caused by the organic acids produced by *Lb. brevis* M30I–2. HPLC analysis of the sourdoughs revealed that in addition to the lactic acid produced by all LAB strains, intense production of acetic acid took place in sourdoughs fermented by *Lb. brevis* M30I–2.

Acetic acid has a dissociation constant of 4.8. As a result, at pH values below 4, as achieved by *Lb. brevis* M30I–2 in our experiments, it is present in the sourdough in an undissociated form. This uncharged form of acid is lipid soluble and may easily diffuse inside the fungal cell membrane and dissociate inside it (Piard and Desmazeaud, 1991). The H+ protons released acidify the cytoplasm and thereby inhibit the action of endogenous enzymes. This forces the cell to waste energy exporting excess protons. In addition, an excess of H+ protons can denature acid sensitive proteins within the microbial cell (Piard and Desmazeaud, 1991; Russell and Diez–Gonzalez 1998; Schnürer and Magnusson, 2005). Finally, high levels of anions that accumulate in the cytoplasm after dissociation of acetic acid can have osmotic effects on the fungal cell (Young and Foegeding, 1993). Mould suppression by different strains of *Lb. brevis*

447 species has previously been described in a number of studies. Thus, nine different strains of *Lb.*
448 *bevis* isolated from sourdoughs and brewing barely were described as producers of proteinaceous
449 compounds and organic acids with wide–spectrum antifungal activity (De Muynck et al., 2004;
450 Gerez et al., 2009; Mauch et al., 2010; Guo et al., 2011) In another study performed by
451 Tatsadjieu, Tchikoua, and Mbofung (2016) two strains of *Lb. brevis* isolated from corn samples
452 demonstrated the ability to inhibit up to 91% of all tested fungal strains during *in vitro* screening.
453 A synergistic effect of organic acids and ethanol produced by heterofermentative *Lb. brevis* was
454 assumed to be a potential cause of this strong inhibiting activity (Tatsadjieu, Tchikoua and
455 Mbofung, 2016).

456 It has been shown that the formation of antifungal compounds can vary depending on the
457 cultivation matrix (Lavermicocca et al., 2000; Stiles et al., 2002). Besides, application of some
458 antifungal compounds in the end product can be limited due to their instability (Pawlowska et al.,
459 2012). Also, previous studies have reported that different species of contaminative fungi could
460 be affected in different ways by the antifungal compounds produced by specific strains of LAB
461 (Lavermicocca et al., 2000; Magnusson and Schnurer 2000; Gerez et al., 2009; Manini et al.,
462 2016). Our *in situ* antifungal assay with the *Lb. brevis* M30I–2 strain revealed that the mould
463 species we tested demonstrated different levels of sensitivity. Thus, the mycelliar form of *A.*
464 *chevalieri* D121 and *P. citrinum* D221 showed the highest sensitivity to compounds produced by
465 *Lb. brevis* M30I–2. *Lb. brevis* M30I–2 was also able to completely inhibit spore germination in
466 all *Aspergilli* and *P. citrinum* D221 species tested. In contrast, the first signs of *P. corylophilum*
467 A111 spore generation appeared during the fourth day of storage. *P. corylophilum* is a widely
468 known bread contaminant and demonstrates low sensitivity to both calcium propionate and
469 sodium benzoate – two chemical compounds commonly used as bread preservatives (Lund et al.,

1996; Lavermicocca et al., 2000). Moreover, Lavermicocca et al (2000) reported that *P. corylophilum* resists the inhibiting compounds produced by the antifungal LAB (*Lactobacillus alimentarius* 5Q, *Lactococcus lactis* subsp. *lactis* 11M and *Leuconostoc citreum* 10M) tested in the study. However, the use of *Lb. brevis* M30I–2 as starter culture prolonging the shelf life of bread contaminated with a *P. corylophilum* spore suspension by up to three days comparing with a control sample.

Conclusions:

Type I rye sourdough processes require starter cultures with multiple functional metabolic properties that contribute to dough acidification, flavor formation, and bio preservation of the end product. Moreover, these cultures should be adapted to the Type I rye sourdough ecosystem, support a high acidity, be robust to changes in fermentation temperature, and provide a reproducible conversion of fermentable carbohydrates. Selection of starter bacteria with a desirable combination of functional properties is a complicated process. In the present study, we examined four lactic acid bacteria strains *Lactobacillus plantarum* M30I–1, *Lactobacillus brevis* M30I–2, *Lactobacillus paralimentarius* M30I–3 and *Lactobacillus cructorum* M30I–9 that were previously isolated from spontaneously started laboratory–scale rye sourdough. Both *Lactobacillus plantarum* M30I–1 and *Lactobacillus brevis* M30I–2 demonstrated a proper acidifying activity at ambient temperatures. However, among all the strains we tested, only *Lactobacillus brevis* M30I–2 was able to end exponential growth phase in 12h in dough fermented at 20°C. In addition, the same strain was able to inhibit the growth of contaminating moulds.

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Table 1. Bacteria used in the study and cultivation conditions applied.

Microorganisms	Origin	Cultivation conditions			
Starter LAB		**Temperature**	**Time, h**	**Media**	**Oxygen**
Lactobacillus plantarum M30I–1 *Lactobacillus. brevis* M30I–2 *Lactobacillus paralimentarius* M30I – 3 *Lactobacillus crustorum* M30I–9	Spontaneously started laboratory Type I rye sourdough	30	24	SDB agar/broth (maltose 20 g l^{-1}, Tween 80 0.3 g l^{-1}, trypticase 5 g l^{-1}, pH5.6)	+
Reference bacteria					
Bacillus cereus ATCC 10876 *Staphylococcus aureus* ATCC 25923 *Yersinia enterocolitica ATCC 27729* *Escherichia coli ATCC 25922*	BIOR microbial collection	37	24	Nutrient agar (Lab M LTD, UK) Brain Heart Infusion Broth (Lab M LTD, UK)	+

Table 2. Fungi used in the study and cultivation conditions applied.

Microorganisms	Origin	Cultivation conditions			
Yeast		**Temperature**	**Time, h**	**Media**	**Oxygen**
Candida humilis SR1	Industrial Type I sourdough	30	24	Yeast Extract Peptone Dextrose (YPD) agar/broth (dextrose 20 g l^{-1}; peptone 20 g l^{-1}; yeast extract 10 g l^{-1})	+
Reference Moulds					
Aspergillus brasiliensis ATCC 16404	BIOR microbial collection	25	168	Sabouraud agar/broth (glucose 40 g l^{-1}, peptone 10 g l^{-1}, agar 20 g l^{-1} pH=5.6)	+
Aspergillus niger B213	Moulded rye bread				
Aspergillus tubingensis B123					
Aspergillus chevalieri D121					
Penicillium corylophilum A111					
Penicillium citrinum D221					

Table 3. Growth parameters: maximum specific growth rate (μ_{max}), maximum heat flow (P_{max}), time at maximum heat production rate (t_{Pmax}) and mean values of bacteria counts, concentrations of lactic and acetic acids, acidity and pH of studied sourdoughs after 12 hours of fermentation at 20 and 30 °C.

	μ_{max}, h^{-1}	t_{Pmax} (h)	P_{max}, $\mu W g^{-1}$	Log(CFUg^{-1})	pH	TTA	Lactate, mmol gDM^{-1}	Acetate, mmol gDM^{-1}
A20	0.127±0.01	>12	NR*	8.73±0.41	4,12±0.04	11.9±0.33	111.91±0.12	22.68±0.30
B20	0.175±0.01	11.5	239.5±0.01	9.14±0.11	4.14±0.05	12.61±0.22	103.38±0.00	102.17±0.40
C20	0.095±0.00	>12	NR	8.45±0.02	4.40±0.02	9.05±0.22	82.01±0.33	ND**
D20	0.121±0.03	>12	NR	8.64±0.00	4.42±0.02	8.93±0.45	50.13±0.12	27.17±0.20
A30	0.472±0.02	1.5	268.6±0.01	9.23±0.16	3.62±0.04	18.16±0.50	260.68±0.09	26.40±0.22
B30	0.354±0.00	3	487.1±0.02	9.68±0.08	3.93±0.07	20.63±0.30	191.93±0.50	109.95±0.22
C30	0.392±0.01	2.0	247.8±0.00	9.10±0.01	3.68±0.14	17.02±0.97	230.65±0.17	25.82±0.33
D30	0.451±0.01	1.5	254.6±0.01	9.20±0.01	3.74±0.06	17.10±0.20	233.44±0.33	26.42±0.33

A – dough inoculated with *Lb. plantarum* M30I–1, B – dough inoculated with *Lb. brevis* M30I–2, C – dough inoculated by *Lb. paralimentarius* M30I–3, *Lb. crustorum* M30I–9.

*NR – not reached

**ND – not detected

Table 4. Volatile compounds detected in experimental sourdoughs after 12 hours of fermentation at 20 °C and 30 °C.

	Compound	A20	A30	B20	B30	C20	C30	D20	D30	Odor
Alcohols	Ethanol	+	+	+	+	+	+	+	+	Alcohol
	2–Methyl–1–butanol	+	+	**ND**	**ND**	+	+	+	+	Wine, fruity
	2,3–Butanediol	+	+	+	+	+	+	+	+	Creamy, buttery
	2–Ethyl–1–hexanol	+	+	+	+	+	+	+	+	Citrus, fresh, floral, oily
	1–Octanol	+	+	+	+	+	+	+	+	Waxy, aldehydic, floral, orange
	1–Dodecanol	+	+	+	+	+	+	+	+	Soapy, waxy, honey
Esters	Ethyl acetate	+	+	+	+	+	+	+	+	Fruity
	Isoamyl acetate	+	+	+	+	+	+	+	+	Ester, ripe fruit
	Hexyl acetate	+	+	+	+	+	+	+	+	Fruity, green, apple
	Phenylethyl acetate	+	+	+	+	+	+	+	+	Floral, rose, sweet, honey
	Propyl decanoate	+	+	**ND**	**ND**	+	+	+	+	Fatty, fruity, waxy, vegetable
	Ethyl undecanoate	+	+	+	+	+	+	+	+	Soapy, waxy, fatty
Aldehydes	Butyrolactone	+	+	+	+	+	+	+	+	Creamy, fatty
	Benzaldehyde	+	+	**ND**	**ND**	+	+	+	+	Strong, sharp, sweet
	(Z)–2–Decenal	**ND**	**ND**	+	+	**ND**	**ND**	**ND**	**ND**	Fatty, orange, aldehydic
	(E,Z)–2,4–Decadienal	+	+	**ND**	**ND**	+	+	+	+	Fried
	(E,E)–2,4–Decadienal	**ND**	**ND**	+	+	**ND**	**ND**	**ND**	**ND**	Fatty, cucumber
Organic acids	Acetic acid	+	+	+	+	+	+	+	+	Pungent, sour
	Butyric acid	+	+	+	+	+	+	+	+	Sharp, acetic, cheese
	2–Ethylhexanoic acid	+	+	**ND**	**ND**	+	+	+	+	Paint–like
	Heptanoic acid	+	+	+	+	+	+	+	+	Rancid, sour, cheesy
	Octanoic acid	+	+	+	+	+	+	+	+	Fatty, waxy, rancid
	Nonanoic acid	+	+	+	+	+	+	+	+	Waxy, cheese, dairy
	Dodecanoic acid	+	+	+	+	+	+	+	+	Fatty

A – dough inoculated with *Lb. plantarum* M30I–1, B – dough inoculated with *Lb. brevis* M30I–2, C – dough inoculated by *Lb. paralimentarius* M30I–3, *Lb. crustorum* M30I–9.

*ND – not detected

Table 5. Total Titratable Acidity (TTA), pH, bacterial and yeast counts in experimental mixed sourdoughs fermented at 20 and 30°C after 14 refreshment cycles.

Starter		pH	TTA	LAB Log($CFUg^{-1}$)	Yeast Log($CFUg^{-1}$)
A + *C. humilis* SR1	20 °C	3.82±0.03	15.67±0.10	9.30±0.50	8.11±0.17
	30 °C	3.74±0.05	18.38±0.11	9.23±0.33	6.60±0.11
B + *C. humilis* SR1	20 °C	3.85±0.09	18.67±0.18	9.48±0.17	7.60±0.34
	30 °C	3.71±0.02	20.82±0.05	9.50±0.65	7.15±0.22
C + *C. humilis* SR1	20 °C	3.90±0.03	15.07±0.07	9.21±0.44	8.42±0.69
	30 °C	3.77±0.03	17.32±0.08	9.13±0.12	7.78±0.21
D + *C. humilis* SR1	20 °C	3.80±0.07	15.53±0.08	9.53±0.28	8.34±0.85
	30 °C	3.73±0.07	18.47±0.02	9.29±0.17	7.56±0.09

A – *Lb. plantarum* M30I–1; B – *Lb. brevis* M30I–2; C – *Lb. paralimentarius* M30I–3; D – *Lb. crustorum* M30I–9

Table 6. Effect of live biomass (B) and cell free supernatant (CFS) on the growth of reference bacteria and mould *in–vitro*.

	Lb. plantarum M30I–1		*Lb. brevis* M30I–2		*Lb. paralimentarius* M30I–3		*Lb. crustorum* M30I–9	
	B	CFS	B	CFS	B	CFS	B	CFS
E. coli ATCC 25922	+++	++	++	+	+++	++	++	++
B. cereus ATCC 10876	+++	+	+++	+++	+++	+	+++	+
S. aureus ATCC 25923	++	+	++	–	+++	+	+++	–
Y. enterocolitica ATCC 27729	+++	++	+++	+	+++	++	+++	++
A. brasiliensis ATCC 16404 spores	–	–	+++	+	–	–	–	–

Antimicrobial activity was interpreted as follows: "–" – no inhibition; "+" – inhibition zone = 0.1 – 0.5cm; "++" – inhibition zone = 0.5 – 1.0cm; "+++" – inhibition zone >1.0cm

Table 7. **The ability of *Lb. brevis* M30I–2 to suppress the growth of mould species specific for rye breads.**

Time after contamination, days	*Penicillium corylophilum* A111		*Penicillium citrinum* D221		*Aspergillus niger* B213		*Aspergillus chevalieri* D121		*Aspergillus tubingensis* B123	
	S	M	S	M	S	M	S	M	S	M
1	–	–	–	–	–	–	–	–	–	–
2	–	+	–	–	–	+	–	–	–	+
3	–	+	–	+	–	+	–	+	–	+
4	+	++	–	+	–	++	–	+	–	+
5	++	++	–	+	–	++	–	+	–	++
7	++	++	–	+	–	++	–	+	–	++

S– breads contaminated with spore suspension. M–breads contaminated with mycelium.

Fungistatic activity was evaluated as follows: "–" – no visual sings of fungal growth; "+" – hyphae formation; "++" – sporulation

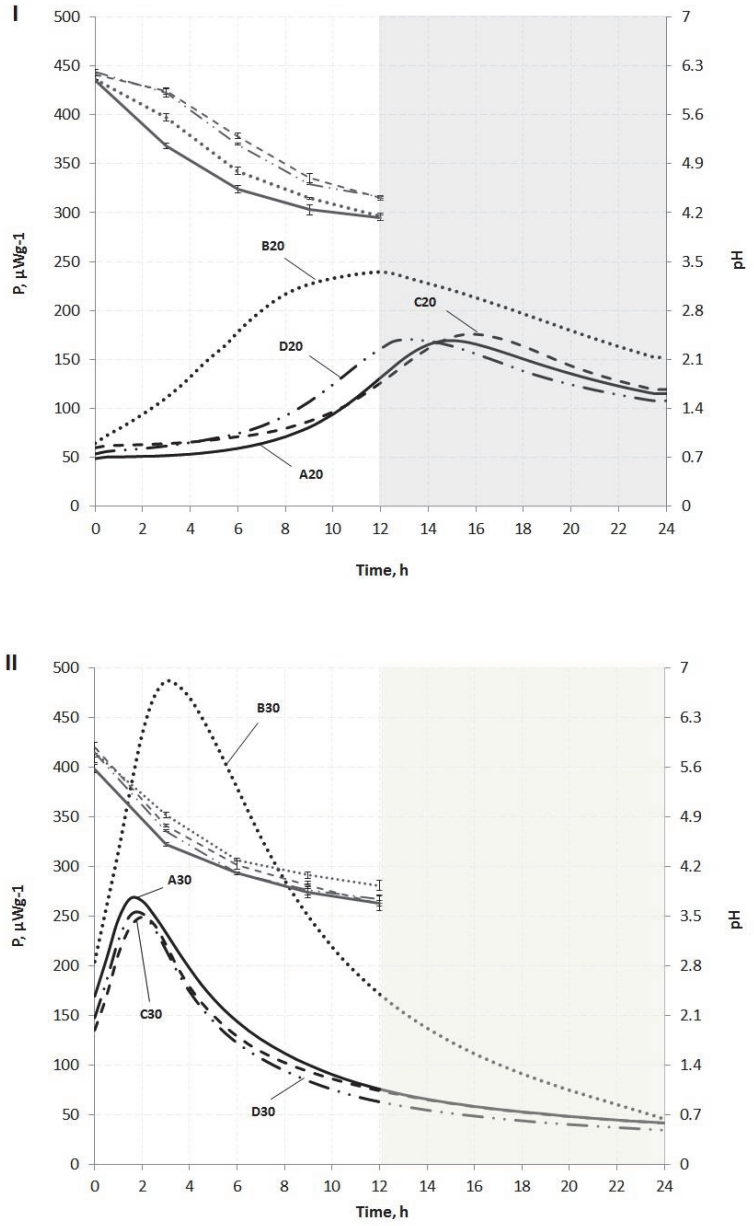

Fig 1. pH change (grey lines) and power time curves (black lines) describing the growth of individual starter bacteria in solid sourdough at 20 °C (I) and 30 °C (II). A – dough

inoculated with *Lb. plantarum* M30I–1, B – dough inoculated with *Lb. brevis* M30I–2, C – dough inoculated by *Lb. paralimentarius* M30I–3, *Lb. crustorum* M30I–9.

PUBLICATION IV

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PCR–based fingerprinting and identification of contaminative fungi isolated from rye breads.

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PCR–based fingerprinting and identification of contaminative fungi isolated from rye breads

M. Surženko[1,2], K. Kontram[1,2] and I. Sarand[2,*]

[1]Competence Center of Food and Fermentation Technologies (CCFFT), Akadeemia tee 15A, EE12618 Tallinn, Estonia
[2]Tallinn University of Technology, Department of Chemistry and Biotechnology, Akadeemia tee 15, EE12618 Tallinn, Estonia
[*]Correspondence: inga.sarand@ttu.ee

Abstract. Fungi are the most frequent cause of microbial spoilage in baked products, including rye bread. As the baking process destroys fungal spores in bread, the post–processing is the main source for mould contamination. Rapid detection methods are needed to track down the origin of the contamination source. In the present research we used a combined molecular approach consisting of PCR–fingerprinting with an M13 primer and further identification of each genotype by amplification and sequencing of the Internal Transcribed Spacer region, the β–tubulin gene and the D1/D2 region of the large subunit of the 28S rDNA. Different rye breads from five bakeries were stored plastic–packed for one month and the fungal colonies with unique morphology were isolated from the bread surfaces. Based on random amplified polymorphic DNA analysis using M13 primer 50 fungal isolates were clustered into eight groups and identified as *Aspergillus chevalieri*, *Aspergillus flavus/oryzae*, *Aspergillus niger*, *Aspergillus tubingensis*, *Penicillium citrinum*, *Penicillium corylophilum, Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* species. Sequencing of the β–tubulin gene and the ITS region showed an equal efficiency for the identification of *Penicillium* species, whereas only the sequence of the β–tubulin gene allowed us to identify most isolates from the genus *Aspergillus* including closely–related black–spored *Aspergillus* species. Yeasts were identified at the species level based on the sequences of the Internal Transcribed Spacer region and the D1/D2 region.

Key words: Mould identification, Yeast identification, PCR–fingerprinting, ITS region, D1/D2 region, β–tubulin gene, rye sourdough breads.

INTRODUCTION

Fungi are the most frequent cause of microbial spoilage in baked products (Legan, 1993; Saranraj & Geetha, 2012). According to Malkki & Rauha (2000) up to 5% of the total bread produced yearly worldwide is lost due to fungal deterioration. Fungal spores are killed by heat treatment during the baking process and thus, contamination occurs post baking through the air and by direct contact with processing equipment during cooling, slicing, and packaging (Knight & Menlove, 1961; Legan, 1993).

Dough acidification is an important step in traditional rye bread technology. Sourdough, a mixture of water and flour fermented by lactic acid bacteria (LAB), is routinely used as acidifier (Kulp & Lorenz, 2003). Low pH (3.5–4.8) of classical rye breads combined with metabolites produced by LAB during fermentation process bio–

protect against some bacterial spoilage (Schnürer & Magnusson, 2005; Gerez et al., 2009; Dalie et al., 2010). Nevertheless, rye breads can be contaminated by a range of different moulds. Species from the *Penicillium* and *Aspergillus* genera seem to predominate (Lund et al., 1996). *Penicillium verrucosum*, *Aspergillus ochraceus*, *Aspergillus bombycis*, and many other representatives of these two genera are well known mycotoxin producers, including aflatoxins B1, B2, G1, G2, and ochratoxin A (Schmidt, 2003; Varga et al., 2011). Dich et al. (1979) found an aflatoxin producing *Aspergillus flavus* in spoiled rye bread and ochratoxigenic *A. ochraceus* was isolated from mouldy bread in Italy (Visconti & Bottalico, 1983). Thus, in addition to economic losses, public health problems cannot be excluded (Legan, 1993; Bento et al., 2009; Duarte et al., 2009; Gerez et al., 2014).

Only a few papers describing contaminative fungi isolated from rye breads have been published and all of them are based only on morphological identification of fungal isolates (Dich et al., 1979; Spicher, 1985; Lund et al., 1996). However, recent studies revealed that phenotypic–based classification of closely–related mould species can be confusing and lead to misidentification (Samson et al., 2007; Silva et al., 2011; Jang et al., 2012). In addition, morphological identification cannot be used for contamination source tracking at strain level. Instead, DNA based methods should be used for it. Information regarding effectiveness of different molecular markers for identification of food contaminating fungi is so far limited (Le Lay et al., 2016; Garnier et al., 2017). Presently, there is no single molecular marker or method advised for fungal identification or genotyping (Vanhee et al., 2010; Araujo, 2014). Sequencing of Internal Transcribed Spacer (ITS) region, D1/D2 region of 28S subunit, β–tubulin, actin, calmodulin and RPB2 gene regions are used for identification (Vanhee et al., 2010; Araujo, 2014).

The aims of our research were to isolate contaminative fungi from rye breads supplied by different Estonian bakeries and to test the effectiveness of different molecular markers in identifying common rye bread contaminative fungi. Discrimination level of random amplified polymorphic DNA (RAPD) analysis using M13 primer followed by sequencing of β–tubulin gene, ITS region and D1/D2 region of 28S subunit for different fungal species was evaluated in order to compose fast and reliable protocol for identification and tracking mould contaminations in baking industry.

MATERIALS AND METHODS

Bread samples and fungi isolation

Ten sliced sourdough rye breads produced by five different Estonian bakeries, designated A, B, C, D and E were purchased. Loaves were made without any preservatives and packed into plastic bags. The presence of fungal colonies on the surface of each loaf was evaluated visually after one, two, three, and four weeks of storage at room temperature. At the end of the fourth week all fungal colonies with different morphology were isolated from the surface of each bread onto Sabouraud Dextrose agar (10.0 g l^{-1} peptone, 12.0 g l^{-1} agar, 40.0 g l^{-1} D–glucose, pH = 5.3). These fungi were then cultivated at 25 °C for 7 days and isolated to obtain monosporal cultures. Pure cultures were cultivated on Yeast Extract Sucrose agar (YES) (yeast extract 20 g l^{-1},

sucrose 150 g l^{-1}, $MgSO_4*7H_2O$ 0.5 g l^{-1}, agar 20 g l^{-1}) at 25 °C for 7 days and stored at + 4 °C for further use.

DNA extraction from fungal cultures

The pure fungal cultures were cultivated in Sabouraud Dextrose broth (10.0 g l^{-1} peptone, 40.0 g l^{-1} D–glucose, pH = 5.3) at 25 °C for 5 days. DNA from the resulting fungal biomass was extracted according to Azevedo et al. (2000).

Random amplified polymorphic DNA analysis using M13 primer

A PCR reaction was performed in 25 µl volumes containing 100 ng of fungal DNA, 40 pmol of M13 primer (5'–GAGGGTGGCGGTTCT–3') (Meyer et al., 1999) (Microsynth AG, Switzerland) and 5 µl of 5x HOT FIREPol® Blend Master Mix (Solis BioDyne, Estonia). The PCR was performed as it was described by Meyer et al. (1999) with some modifications. Initial hold at 95 °C for 15 minutes was followed by 35 cycles of 94 °C for 20 seconds, 50 °C for 1 minute, and 72 °C for 20 seconds with a final extension at 72 °C for 6 minutes. The amplified DNA was then analyzed on 15 g kg^{-1} agarose gel at 70 V for 45 minutes and visualized under UV light.

Identification of fungal isolates

The ITS region was amplified using ITS1 (5'–TCCGTCGGTGAACCTGCGG–3') and ITS4 (5'–TCCTCCGCTTATTGATATGC–3') (Kwiatkowski et al., 2012) (Microsynth AG, Switzerland) primer pair. D1/D2 region of the 28S rDNA was amplified using forward primer NL–1 (5′–GCATATCAATAAGCGGAGGAAAAG–3') and reverse primer NL–4 (5′–GGTCCGTGTTTCAAGACGG–3') (Kwiatkowski et al., 2012) (Microsynth AG, Switzerland). For each primer set PCR reactions were performed in 50 µl volumes containing 100 ng of fungal DNA, 40 pmol of each primer, and 10 µl of 5x HOT FIREPol®Blend Master Mix (Solis BioDyne, Estonia) with the remaining volume consisting of ultrapure water. Amplifications of ITS region and D1/D2 region were performed according to Kwiatkowski et al. (2012) with minor modifications. Amplification of ITS region was performed with initial denaturation at 95 °C for 15 minutes followed by 30 cycles of 95 °C for 30 seconds, 55 °C for 1 minute and 72 °C for 1 minute and a final extension at 72 °C for 6 minutes. For the amplification of D1/D2 region after initial denaturation at 95 °C for 15 minutes 30 cycles of 94 °C for 15 seconds, 55 °C for 30 seconds and 68 °C for 2 minutes with a final extension at 68 °C for 5 minutes were performed.

The β–tubulin gene sequences were amplified using Bt2a (5'– GGTAACCAAATCGGTGCTGCTTTC–3') and Bt2b (5'– ACCCTCAGTGTAGTGACCCTTGGC–3') (Zampieri et al., 2009) primer pair (Microsynth AG, Switzerland). PCR protocol was modified from Silva et al. (2011). The 50 µl of PCR mixture contained 20 ng of fungal DNA, 40 pmol of each primer, and 10 µl of 5x HOT FIREPol®Blend Master Mix (Solis BioDyne, Estonia) with the remaining volume consisting of ultrapure water. The mixture was subjected to the following amplification program: initial hold at 95 °C for 15 minutes; followed by 35 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 90 seconds; followed by a final extension at 72 °C for 5 minutes.

PCR products were purified with GeneJET PCR Purification kits (Thermo ScientificTM, Tartu, Estonia) and sequencing of the fragments was carried out at a commercial facility (Estonian Biocenter, Tartu, Estonia). The sequences obtained were compared with the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, USA).

RESULTS AND DISCUSSION

The growth of fungal colonies on sourdough rye breads obtained from bakeries B, C and D was observed during the first week of storage, whereas breads from bakeries A and E got spoiled at the end of third and fourth week, respectively (Table 1). In total, 50 colonies were picked up from studied breads and the monosporal cultures were obtained. Detected fungal morphological biodiversity varied among breads produced by different bakeries. Two fungal morphotypes were detected on A_{bakery} (A1, A111) and C_{bakery} breads (C111, C121) and three morphotypes on breads from B_{bakery} (B111, B123, B213) (Fig. 1). The most morphologically diverse fungal community was observed on D_{bakery} breads (D131, D121, D221, D231, Fig. 1), whereas in samples from E_{bakery} only one type of fungi was detected (E1, Fig. 1).

Table 1. Identified fungal species in bread samples from five Estonian bakeries (A, B, C, D and E) and the duration of mould–free shelf life

Representatives of isolated fungal strains	RAPD pattern	Presence in fungal population of each bakery				
		A	B	C	D	E
Penicillium corylophilum A111, C111	RAPD I	+	–	+	–	–
Wickerhamomyces anomalus A1	RAPD II	+	–	–	–	–
Aspergillus niger B213, D231	RAPD III	–	+	–	+	–
Aspergillus flavus/oryzae B111, D131	RAPD IV	–	+	–	+	–
Aspergillus tubingensis B123, C121	RAPD V	–	+	+	–	–
Aspergillus chevalieri D121	RAPD VI	–	–	–	+	–
Saccharomyces cerevisiae E1	RAPD VII	–	–	–	–	+
Penicillium citrinum D221	RAPD VIII	–	–	–	+	–
Mould–free shelf life, weeks		< 3	< 1	< 1	< 1	> 3

Most colonies isolated from A_{bakery} breads in the end of the storage period were of the white variety and grew to about 4 cm in diameter on YES agar without any clear signs of sporulation (A111, Fig. 1). A minority of isolates produced smaller colonies on YES agar without forming mycelium (A1, Fig. 1). Upon microscopic examination of A1 type isolates we detected the presence of budding yeast–like cells.

Moulds isolated from B_{bakery} breads produced large pale – yellow colonies with yellow and white outlines (B111, Fig. 1) or large ivory colonies with pale– and dark–brown conidia respectively and rich sporulation (B123, B213, Fig. 1). A part of moulds isolated from C_{bakery} breads created colonies similar to A111 isolates (C111, Fig. 1), whereas others formed large ivory colonies with pale–brown spores in the middle and were rather similar to B123 isolates (C121, Fig. 1). Moulds isolated from D_{bakery} breads produced large pale–yellow colonies similar to B111 isolates (D131, Fig. 1), small (about 15 mm in diameter) white colonies with grey and yellow edges (D121, Fig. 1), small white–grey colonies without any signs of sporulation (D221, Fig. 1) and large

ivory colonies with dark–brown conidia in the middle similar to B213 isolates (D231, Fig. 1). Finally, fungi isolated from E_{bakery} breads created small ivory colonies with smooth matte surface on YES agar (E1, Fig. 1) and their microscopic examination revealed yeast–like cells.

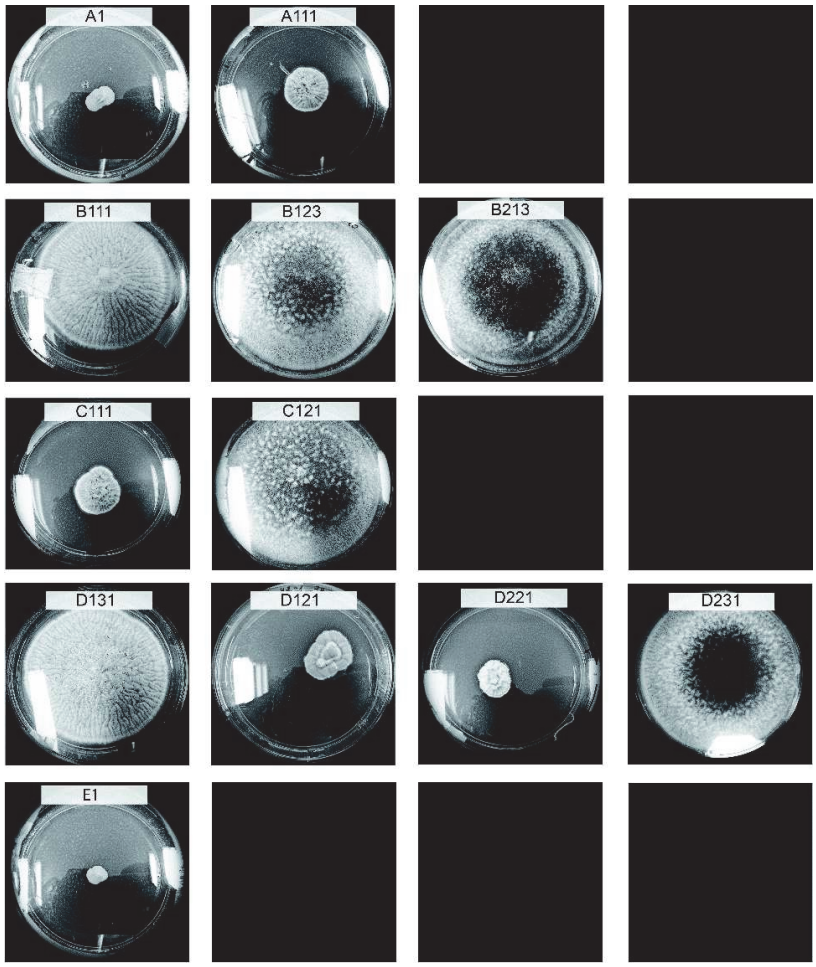

Figure 1. Fungal morphotypes obtained from breads originating from bakeries A (A1; A111); B (B111; B123; B213); C (C111; C121); D (D131; D121; D221; D231) and E (E1).

All 50 fungal isolates were genotyped by RAPD–PCR using M13 primer. Eight reproducible RAPD patterns with clear banding profiles were obtained (Fig. 2). Several RAPD patterns were bakery–specific (RAPD II, RAPD VI–VIII, Table 1), whereas representatives of other RAPD patterns were found on breads produced by different bakeries (Table 1). It is considered that biodiversity of contaminating fungi is related to the frequency of fungal infections in bakeries (Lund et al., 1996). Indeed, in our study the direct correlation between the numbers of moulds with different RAPD patterns and duration of shelf–life of rye bread was observed. Samples from A_{bakery} and E_{bakery}, where only yeasts or one mould species were detected, had the longest shelf–life among all bread samples evaluated in this study (Table 1).

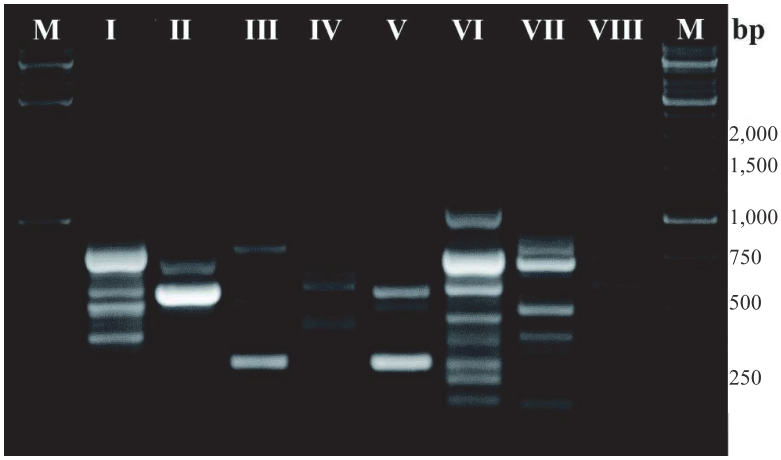

Figure 2. Eight RAPD patterns of fungal isolates obtained by RAPD–fingerprinting with M13 praimer (see the Table 1 for more detailed information). M: 1 kb DNA ladder.

The representative isolates of different RAPD types were identified based on sequences of the ITS region, the β–tubulin gene, or the D1/D2 region of the large subunit of the 28S rDNA as *Aspergillus chevalieri, Aspergillus flavus/oryzae, Aspergillus niger, Aspergillus tubingensis, Penicillium citrinum, Penicillium corylophilum, Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* species (Annex 1). None of the molecular markers used were suitable for identification of all fungal isolates either due to low discriminative capacity towards a certain species or a continuous failure of amplification/low quality of sequences (Annex 1). In case of isolates belonged to RAPD pattern I sequencing of the β–tubulin gene and ITS region yielded 100% identical sequence data for *Penicillium corylophilum*/*Penicillium obscurum* or *Penicillium corylophilum/Penicillium obscurum/Penicillium chloroleucon* respectively (Annex 1). All identified species belonged to *Penicillium* section *Exilicaulis*. However, as the result of the recent revision of this section by Visagie et al. (2016), both *P. obscurum* and *P. chloroleucon* were considered as synonyms for *P. corylophilum* species. Thus, RAPD I isolates can be identified as *P. corylophilum* based on sequences of β–tubulin gene and

ITS region. This mould species is widely found in cereals and damp buildings (Magan, Arroyo & Alfred, 2003; McMullin, Nsiama & Miller, 2014) and has been previously reported as dominant species in contaminated rye breads made without chemical preservatives (Lund et al., 1996).

Another species of genera *Penicillium* was represented by isolates belonging to RAPD VIII pattern and was identified as *Penicillium citrinum* by all three molecular markers used (Annex 1). There is no data concerning the contamination of rye bread by *P. citrinum* species. However, *P. citrinum* was found in wheat flour and bread in the USA (Bullerman & Hartung, 1973). This species is widely found in soil and plants (Houbraken & Samson, 2011), and thus it may contaminate the bakery environment via flour particles that spread through the air and also by landing on equipment used for slicing and packaging.

Representatives of RAPD III, RAPD IV, RAPD V and RAPD VI patterns belong to the *Aspergillus* genera and were identified at species level only based on β–tubulin gene sequence (Annex 1) including closely–related black–spore *Aspergilli* species *Aspergillus tubingensis* (RAPD V) and *Aspergillus niger* (RAPD III). The latter are the most difficult groups to identify using morphology–based methods (Varga et al., 2000; Varga, Frisvad & Samson, 2011; Jang et al., 2012). Whereas *A. niger* is frequently reported as bread contaminant (Legan, 1993; Lund et al., 1996; Saranraj & Geetha, 2012), there is no data regarding the contamination of rye bread by *A. tubingensis.* RAPD VI pattern was composed by *Aspergillus chevalieri*, a xerophilic mould growing on food with water activity down to 0.65 like rolled oats, chocolate, some dried fruits and nuts (Pomeranz, 1991). Its telemorph, *Eurotium chevalier*, has been detected as a contaminant of milk bread rolls (Le Lay et al., 2016). RAPD IV group also belonged to *Aspergillus* genera but it was not identified at species level. Sequences of both β–tubulin gene and D1/D2 region have the similar identity to the sequences of both *Aspergillus flavus* and *Aspergillus oryzae* species (Annex 1). These are very closely–related species, which are genetically almost identical (Chang & Ehrlich, 2010; Amaike & Keller, 2011) but have different economic impact. While most *A. flavus* strains are aflatoxigenic and infect preharvest and postharvest seed crops, representatives of *A. oryzae* species have been widely used for preparation of traditional fermented foods and beverages. Genome sequence data supports the view that *A. flavus* and *A. oryzae* are the same species with the latter representing a domesticated clade of *A. flavus* (Amaike & Keller, 2011). Although Nikkuni et al. (1998) showed that these two species could be distinguished based on ITS region sequence, Jang et al. (2012) reported that sequences of all targeted regions (ITS, D1/D2 region and β–tubulin gene) were not variable enough to distinguish *A. flavus* from *A. oryzae.*

Isolated morphotypes A1 and E1, which were microscopically identified as yeasts, clustered into RAPD II and RAPD VII patterns, respectively (Table 1). Representatives of RAPD II pattern were identified as *Wickerhamomyces anomalus* based on sequences of ITS and D1/D2 regions while amplification of β–tubulin gene repeatedly failed. All three selected primer pairs performed equally well for the identification of RAPD VII as *Saccharomyces cerevisiae* (Annex 1). In the study performed by Lund et al. (1996) significant part of fungi isolated from spoiled rye breads belonged to yeast species causing surface spoilage of baked products known as ‘chalk moulds’. In our trial yeasts were isolated only from samples obtained from bakeries A and E. Whereas only single cases of contamination by *S. cerevisiae* have been previously described (Spicher, 1985),

Wickerhamomyces anomalus together with *Endomyces fibuliger* and *Hyphopichia burtonii* yeast species belongs to the most frequently reported cause of 'chalk mould' bread defect (Lund et al., 1996; Deschuyffeleer et al., 2011).

Although the ITS region is considered as the universal barcode for fungal identification (Schoch et al., 2012) in our study its discriminative capacity was insufficient for identification of most fungal isolates belonging to *Aspergillus* genera. Garnier with co–authors (2017) noticed its limited taxonomic resolution for *Penicillium* and *Cladosporium* species. Thus, the β–tubulin gene should be recommended as a primary molecular marker for identification of fungi associated with rye breads. Preliminary clustering of fungal isolates with RAPD–PCR appeared to be an efficient way to reduce the sequencing expenses. Additional studies should be performed to evaluate the efficiency of RAPD–PCR fingerprinting with M13 primer to track fungi contamination source at strain level.

CONCLUSIONS

1. Fungi isolated from mouldy rye breads mainly belonged to *Aspergillus* and *Penicillium* species.
2. The β–tubulin gene sequence has higher taxonomic resolution for identification of mould isolates belonging to *Aspergillus* genera comparing to ITS region and D1/D2 region.
3. Clustering of fungal isolates with RAPD–PCR using M13 primer followed by identification based on the β–tubulin gene sequence can be recommended as a protocol for identification and tracking mould contamination in baking industry.

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