

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 Simm³, Tiina Talve¹, Anu Aaspõllu³, 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



OPEN ACCESS

Citation: Viiard E, Bessmeltseva M, Simm J, Talve T, Aaspõllu A, Paalme T, et al. (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

Editor: Danilo Ercolini, University of Naples Federico II, ITALY

Received: July 2, 2015

Accepted: January 15, 2016

Published: February 5, 2016

Copyright: © 2016 Viiard et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: Pyrosequencing data is available in GenBank database under accession numbers KM972414–KM972548.

Funding: Financial support was provided by the European Regional Development Fund (<http://www.struktuurifondid.ee/euroopa-regionaalarengu-fond/>) project EU29994 (EV, MB, TT, IS) together with funds from Estonian Research Council (<http://www.etag.ee/rahastamine/etf-grandid/>) research grant ETF9417 (IS), Ministry of Education and Research (<http://www.etag.ee/rahastamine/institutsionaalne-uurimistoetus/>) research grant IUT19-27 (TP, IS) and the European

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 backslipping 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.

Social Fund Doctoral Studies and Internationalization Programme DoRa (<http://adm.archimedes.ee/stipendiumid/programm-dora/>) administered by SA Archimedes (EV, MB). This work has been partially supported by graduate school „Functional materials and technologies“ receiving funding from the European Social Fund (<http://www.strukturifondid.ee/euroopa-sotsiaalfond-2/>) under project 1.2.0401.09-0079 in Estonia (EV). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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		

doi:10.1371/journal.pone.0148325.t001

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 dH₂O 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 $5000 \times g$. 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 16S 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

doi:10.1371/journal.pone.0148325.t002

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 $8.95 \pm 0.13 \log \text{CFU g}^{-1}$) and B_{bakery} (on average $8.67 \pm 0.32 \log \text{CFU g}^{-1}$), 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: $6 \log \text{CFU g}^{-1}$) 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 $8.47 \pm 0.54 \log \text{CFU g}^{-1}$) 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.

	<i>Bacteroides</i> sp.	<i>Flavobacteriaceae bacterium</i>	<i>Lactobacillus amylovorus</i>	<i>Lactobacillus casei/paracasei</i>	<i>Lactobacillus coleohominis</i>	<i>Lactobacillus fermentum</i>	<i>Lactobacillus frumenti</i>	<i>Lactobacillus helveticus</i>	<i>Lactobacillus panis</i>	<i>Lactobacillus plantarum</i>	<i>Lactobacillus pontis</i>	<i>Lactobacillus rhamnosus</i>	<i>Lactobacillus sanfranciscensis</i>	<i>Lactobacillus secaliphilus</i>	<i>Lactobacillus vaginalis</i>	<i>Lactobacillus zymae</i>	<i>Leuconostoc lactis</i>	<i>Pantoea</i> sp.	<i>Pediococcus acifilactici</i>	
A _{bakery}	A0	–/–	–/–	–/–	–/–	–/+	–/–	–/–	+/+	+/+	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
	A1	–/–	–/–	–/–	+/-	–/–	–/–	–/–	+/+	-/+	–/–	-/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
	A2	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	–/–	-/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
	A3	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	–/–	+/+	–/–	–/–	–/–	–/–	+/-	–/–	–/–	–/–
	A4	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	–/–	+/+	–/–	–/–	–/–	–/–	-/+	–/–	–/–	–/–
B _{bakery}	Bs	–/–	–/–	+/+	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/+	–/–	–/–	–/–	–/–	–/–
	B0	–/–	–/–	+/+	–/–	–/–	–/–	+/+	–/–	+/-	–/–	-/+	–/–	–/–	–/+	–/–	–/–	–/–	–/–	–/–
	B1	–/–	–/–	+/+	–/–	–/–	–/–	+/+	–/–	+/-	–/–	–/–	–/–	–/–	–/+	–/–	–/–	–/–	–/–	–/+
	B2	-/+	-/+	+/+	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/+	–/–	–/–	–/–	–/–	–/–
	B3	–/–	–/+	+/+	–/–	–/–	–/–	+/+	–/–	+/-	–/–	-/+	–/–	–/–	–/+	–/–	–/–	–/–	–/–	–/–
C _{bakery}	B4	–/–	–/–	+/+	–/–	–/–	–/–	+/+	–/–	+/-	–/–	–/–	–/–	–/+	–/–	–/–	–/–	–/–	–/–	–/–
	C0	–/–	–/–	–/–	+/-	–/–	–/–	–/–	–/–	+/-	-/+	+/-	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
	C1	–/–	–/–	–/–	+/-	–/–	+/-	–/–	–/–	–/–	-/+	–/–	-/+	–/–	–/–	+/-	+/-	–/–	+/-	–/–
	C2	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	-/+	–/–	–/–	+/-	–/–	–/–	–/–	–/–
D _{bakery}	C3	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/-	–/–	-/+	–/–	–/–	+/-	–/–	–/–	–/–	–/–
	D1	–/–	–/–	–/–	–/–	–/–	–/–	–/+	–/–	–/–	-/+	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–
	D2	–/–	–/–	–/–	–/–	–/–	–/–	–/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	+/-	–/–	–/–	–/–	–/–
	D3	–/–	–/–	–/–	–/–	–/–	–/–	–/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	+/-	–/–	–/–	–/–	–/–
	D4	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	+/-	–/–	–/–	–/–	–/–
D5	N/ –	N/ –	N/ –	N/ –	N/ –	N/ –	N/ –	N/ +	N/ –	N/ –	N/ +	N/ –	N/ –	N/ –	N/ –	N/ +	N/ –	N/ –	N/ –	N/ –

doi:10.1371/journal.pone.0148325.t003

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.

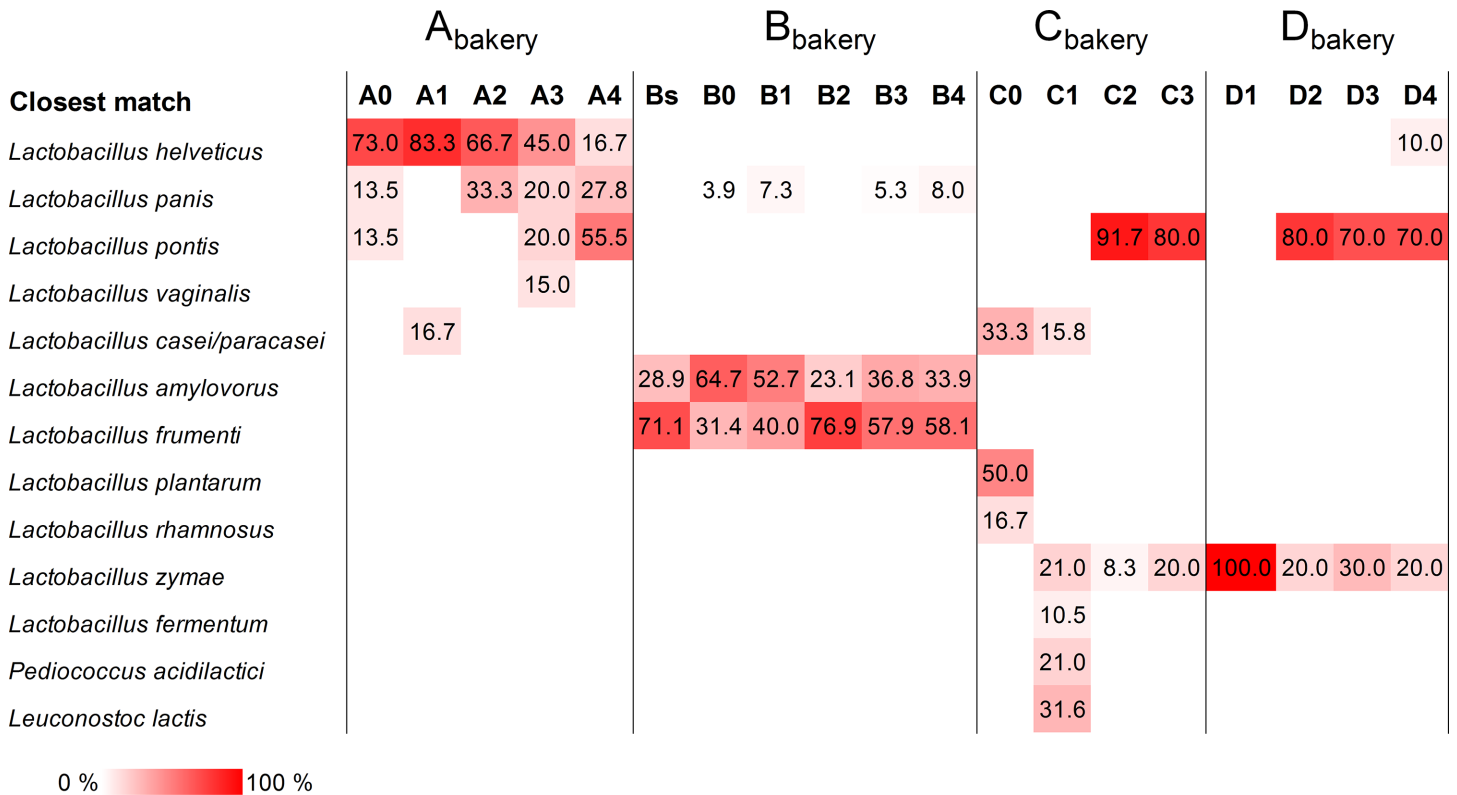


Fig 1. Composition of lactic acid bacterium species, expressed in percentage of the total number of isolates, in rye sourdoughs from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}). Samples are coded according to the description reported in Table 1.

doi:10.1371/journal.pone.0148325.g001

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

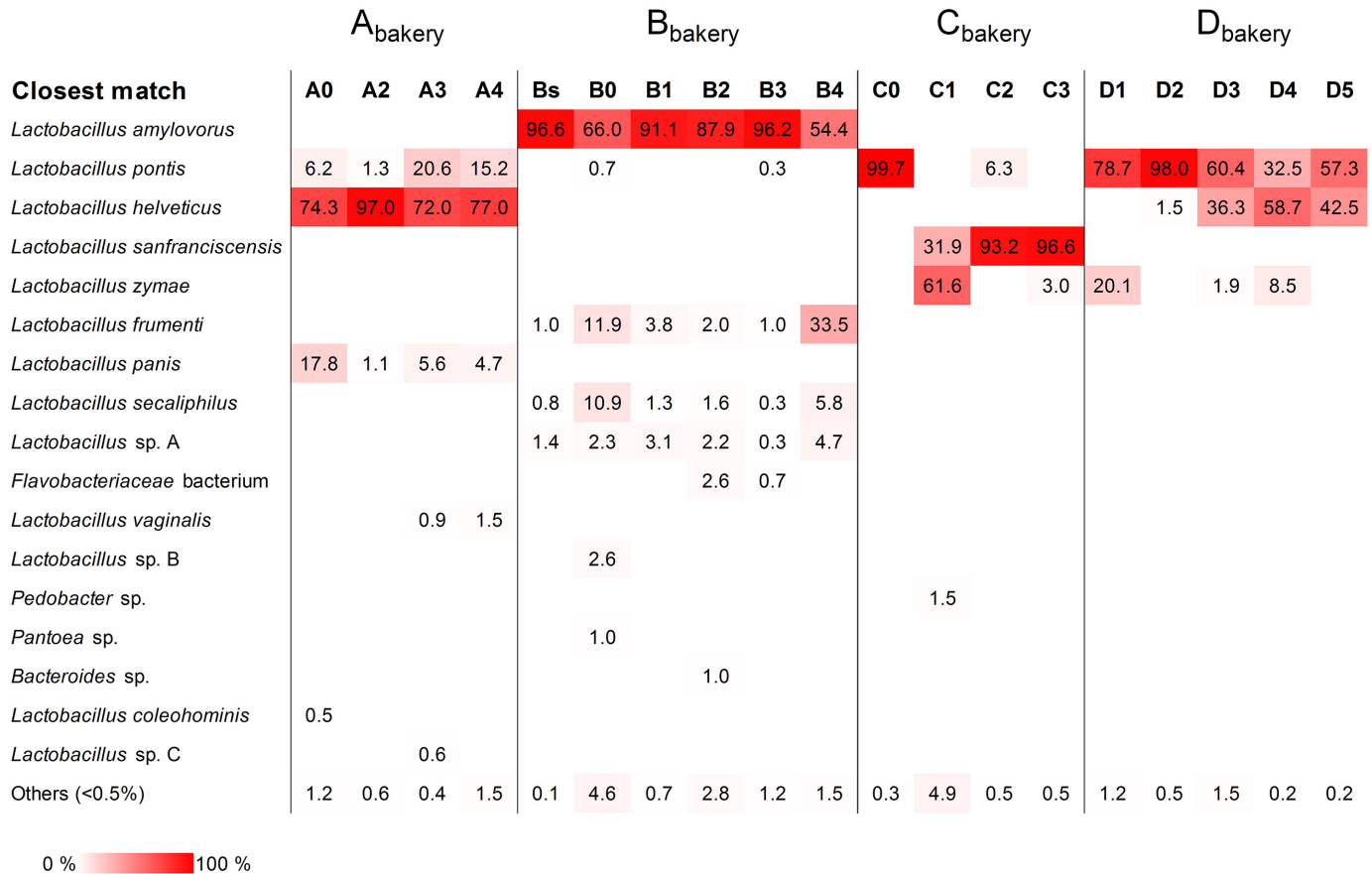


Fig 2. Relative abundance of bacterial species/genera detected in rye sourdoughs from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}) using pyrosequencing of 16S rRNA gene amplicons. Samples are coded according to the description reported in Table 1.

doi:10.1371/journal.pone.0148325.g002

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

doi:10.1371/journal.pone.0148325.t004

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}.

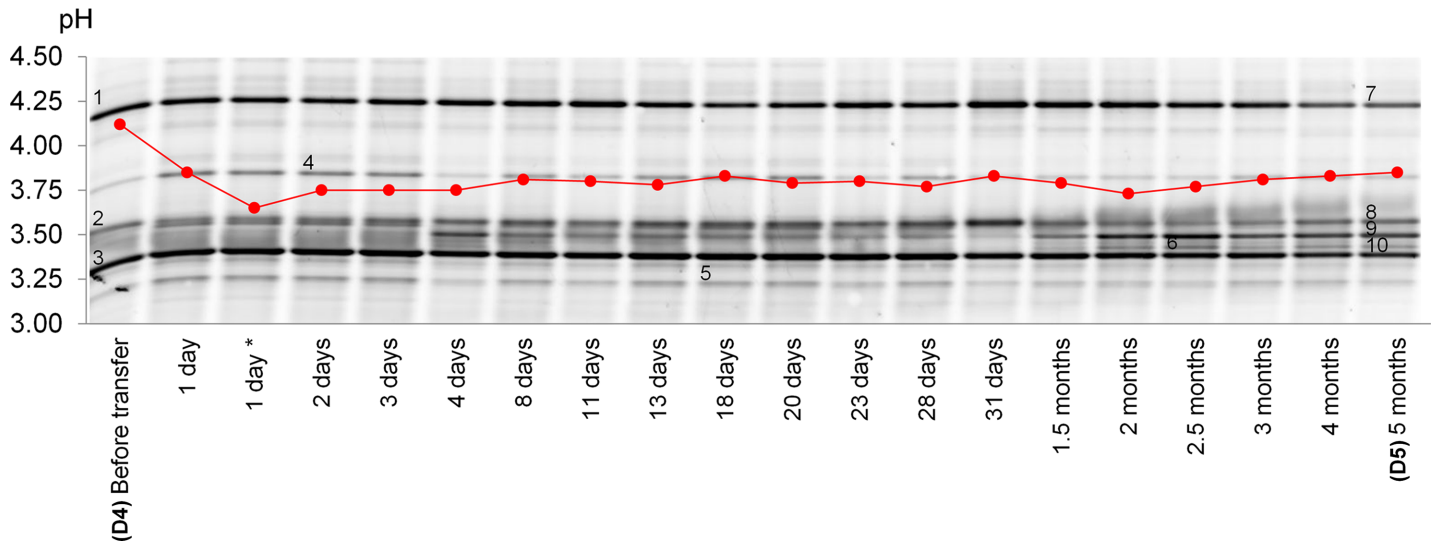


Fig 3. pH (red plot) and lactic acid bacterium species detected by DGGE analysis of the 16S rRNA gene amplicons in rye sourdoughs collected at the D_{bakery} before (D4) and after applying the new propagation protocol. Time after transfer is indicated below the gel (d-day; m-month). Bands: 1, 7 – *Lactobacillus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

doi:10.1371/journal.pone.0148325.g003

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

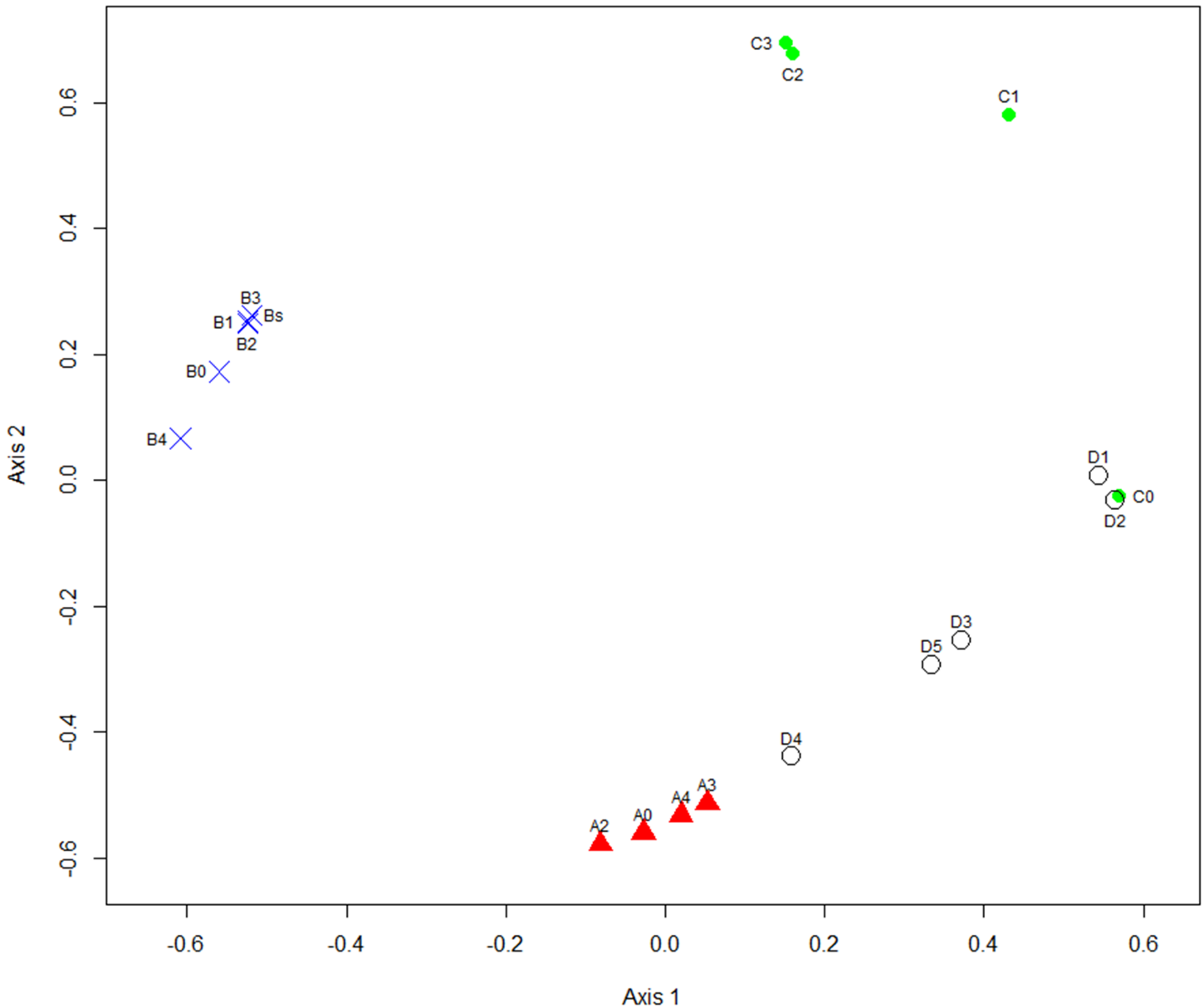


Fig 4. Two dimensional non-metric multidimensional scaling (NMDS) of sourdough samples from four Estonian bakeries (A_{bakery}, B_{bakery}, C_{bakery} and D_{bakery}).

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

The authors would like to thank the four bakeries for providing the sourdough samples.

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.

References

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

2. Hansen AS. Sourdough bread. In: Hui YH, Meunier-Goddik L, Hansen AS, Josephsen J, Nip WK, Stanfield PS, Toldra F, editors. Handbook of Food and Beverage Fermentation Technology. New York: Marcel Dekker, Inc.; 2004. pp. 840–870.
3. Gobbetti M, Corsetti A, Rossi J. The sourdough microflora. Interactions between lactic acid bacteria and yeasts: metabolism of amino acids. World J Microbiol Biotechnol. 1994; 10: 275–279. doi: [10.1007/BF00414862](https://doi.org/10.1007/BF00414862) PMID: [24421010](https://pubmed.ncbi.nlm.nih.gov/24421010/)
4. De Vuyst L, Van Kerrebroeck S, Harth H, Huys G, Daniel H, Weckx S. Microbial ecology of sourdough fermentations: Diverse or uniform? Food Microbiol. 2014; 37: 11–29. doi: [10.1016/j.fm.2013.06.002](https://doi.org/10.1016/j.fm.2013.06.002) PMID: [24230469](https://pubmed.ncbi.nlm.nih.gov/24230469/)
5. Müller M, Wolfrum G, Stolz P, Ehrmann M, Vogel R. Monitoring the growth of *Lactobacillus* species during rye flour fermentation. Food Microbiol. 2001; 18: 217–227.
6. Rosenquist H, Hansen A. The microbial stability of two bakery sourdoughs made from conventionally and organically grown rye. Food Microbiol. 2000; 17: 241–250.
7. Böcker G, Stolz P, Hammes W. Neue Erkenntnisse zum ökosystem sauteig und zur physiologie des sauteig typischen stämme *Lactobacillus sanfrancisco* und *Lactobacillus pontis*. Getreide mehl und brot. 1995; 49: 370–374.
8. Hammes W, Gänzle M. Sourdough breads and related products. In: Woods B, editor. Microbiology of Fermented Foods. London: Blackie Academic/Professional; 1998. pp. 199–216.
9. Meroth C, Walter J, Hertel C, Brandt M, Hammes W. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol. 2003; 69: 475–482. PMID: [12514030](https://pubmed.ncbi.nlm.nih.gov/12514030/)
10. Siragusa S, Di Cagno R, Ercolini D, Minervini F, Gobbetti M, De Angelis M. Taxonomic structure and monitoring of the dominant population of lactic acid bacteria during wheat flour sourdough type I propagation using *Lactobacillus sanfranciscensis* starters. Appl Environ Microbiol. 2009; 75: 1099–1109. doi: [10.1128/AEM.01524-08](https://doi.org/10.1128/AEM.01524-08) PMID: [19088320](https://pubmed.ncbi.nlm.nih.gov/19088320/)
11. Minervini F, Lattanzi A, De Angelis M, Di Cagno R, Gobbetti M. Influence of artisan bakery- or laboratory-propagated sourdoughs on the diversity of lactic acid bacterium and yeast microbiotas. Appl Environ Microbiol. 2012; 78: 5328–5340. doi: [10.1128/AEM.00572-12](https://doi.org/10.1128/AEM.00572-12) PMID: [22635989](https://pubmed.ncbi.nlm.nih.gov/22635989/)
12. Minervini F, De Angelis M, Di Cagno R, Gobbetti M. Ecological parameters influencing microbial diversity and stability of traditional sourdough. Int J Food Microbiol. 2014; 171: 136–146. doi: [10.1016/j.ijfoodmicro.2013.11.021](https://doi.org/10.1016/j.ijfoodmicro.2013.11.021) PMID: [24355817](https://pubmed.ncbi.nlm.nih.gov/24355817/)
13. Scheirlinck I, Van der Meulen R, Van Schoor A, Vancanneyt M, De Vuyst L, Vandamme P et al. Influence of geographical origin and flour type on diversity of lactic acid bacteria in traditional Belgian sourdoughs. Appl Environ Microbiol. 2007; 73: 6262–6269. PMID: [17675431](https://pubmed.ncbi.nlm.nih.gov/17675431/)
14. Valjakka TT, Kerojoki H, Katina K. Sourdough Bread in Finland and Eastern Europe. In: Kulp K, Lorenz K, editors. Handbook of Dough Fermentations. New York: Marcel Dekker, Inc., 2003. pp. 269–297.
15. Kline L, Sugihara T, Miller MW. Microorganisms of the San Francisco sour dough bread process. II. Isolation and characterization of undescribed bacterial species responsible for the souring activity. Appl Microbiol. 1971; 21: 459–465. PMID: [5553285](https://pubmed.ncbi.nlm.nih.gov/5553285/)
16. Viard E, Mihhalevski A, Rühka T, Paalme T, Sarand I. Evaluation of the microbial community in industrial rye sourdough upon continuous back-slopping propagation revealed *Lactobacillus helveticus* as the dominant species. J Appl Microbiol. 2013; 114: 404–412. doi: [10.1111/jam.12045](https://doi.org/10.1111/jam.12045) PMID: [23082800](https://pubmed.ncbi.nlm.nih.gov/23082800/)
17. Frank J, Reich C, Sharma S, Weisbaum J, Wilson B, Olsen G. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl Environ Microbiol. 2008; 74: 2461–2470. doi: [10.1128/AEM.02272-07](https://doi.org/10.1128/AEM.02272-07) PMID: [18296538](https://pubmed.ncbi.nlm.nih.gov/18296538/)
18. Weisburg W, Barns S, Pelletier D, Lane D. Ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991; 173: 697–703. PMID: [1987160](https://pubmed.ncbi.nlm.nih.gov/1987160/)
19. Gafan G, Spratt D. Denaturing gradient gel electrophoresis gel expansion (DGGE)–an attempt to resolve the limitations of co-migration in the DGGE of complex polymicrobial communities. FEMS Microbiol Lett. 2005; 253: 303–307 PMID: [16257140](https://pubmed.ncbi.nlm.nih.gov/16257140/)
20. Meroth C, Hammes W, Hertel C. Identification and population dynamics of yeasts in sourdough fermentation processes by PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol. 2003; 69: 7453–7461. ÖIGE PMID: [14660398](https://pubmed.ncbi.nlm.nih.gov/14660398/)
21. McKenna P, Hoffmann C, Minkah N, Aye P, Lackner A, Liu Z et al. The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis. PLoS Pathog. 2008; 4(2). doi: [10.1371/journal.ppat.0040020](https://doi.org/10.1371/journal.ppat.0040020)
22. Armougom F, Raoult D. Exploring microbial diversity using 16S rRNA high-throughput methods. J Comput Sci Syst Biol. 2009; 2: 74–92.

23. Schloss P, Westcott S, Ryabin T, Hall J, Hartmann M, Hollister EB et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol.* 2009; 75: 7537–7541. doi: [10.1128/AEM.01541-09](https://doi.org/10.1128/AEM.01541-09) PMID: [19801464](https://pubmed.ncbi.nlm.nih.gov/19801464/)
24. Schloss P, Gevers D, Westcott S. Reducing the Effects of PCR Amplification and Sequencing Artifacts on 16S rRNA-Based Studies. *PLoS ONE.* 2011; 6. doi: [10.1371/journal.pone.0027310](https://doi.org/10.1371/journal.pone.0027310)
25. Prusse E, Quast C, Knittel K, Fuchs B, Ludwig W, Peplies J et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 2007; 35: 7188–7196. PMID: [17947321](https://pubmed.ncbi.nlm.nih.gov/17947321/)
26. Yue J, Clayton M. A similarity measure based on species proportions. *Commun Stat Theor Methods.* 2005; 34: 2123–2131.
27. Ehrmann MA, Brandt M, Stolz P, Vogel RF, Korakli M. *Lactobacillus secaliphilus* sp. nov., isolated from type II sourdough fermentation. *Int J Syst Evol Microbiol.* 2007; 57: 745–750. PMID: [17392199](https://pubmed.ncbi.nlm.nih.gov/17392199/)
28. De Vuyst L, Avonts L, Hoste B, Vancanneyt P, Swings J, Callewaert R. The lactobin A and amylovorin L471 encoding genes are identical, and their distribution seems to be restricted to the species *Lactobacillus amylovorus* that is of interest for cereal fermentations. *Int J Food Microbiol.* 2004; 90: 93–106. PMID: [14672834](https://pubmed.ncbi.nlm.nih.gov/14672834/)
29. Leroy F, De Winter T, Adriany T, Neysens P, De Vuyst L. Sugars relevant for sourdough fermentation stimulate growth of and bacteriocin production by *Lactobacillus amylovorus* DCE 471. *Int J Food Microbiol.* 2007; 112: 102–111.
30. De Vuyst L, Schrijvers V, Paramithiotis S, Hoste B, Vancanneyt M, Swings J et al. The biodiversity of lactic acid bacteria in Greek traditional wheat sourdoughs is reflected in both composition and metabolite formation. *Appl Environ Microbiol.* 2002; 68: 6059–6069. PMID: [12450829](https://pubmed.ncbi.nlm.nih.gov/12450829/)
31. Vancanneyt M, Neysens P, De Wachter M, Engelbeen K, Snauwaert C, Cleenwerck I et al. *Lactobacillus acidifarinae* sp. nov. and *Lactobacillus zymae* sp. nov., from wheat sourdoughs. *Int J Syst Evol Microbiol.* 2005; 55: 615–620. PMID: [15774633](https://pubmed.ncbi.nlm.nih.gov/15774633/)
32. Scheirlinck I, Van der Meulen R, De Vuyst L, Vandamme P, Huys G. Molecular source tracking of predominant lactic acid bacteria in traditional Belgian sourdoughs and their production environments. *J Appl Microbiol.* 2009; 106: 1081–1092. doi: [10.1111/j.1365-2672.2008.04094.x](https://doi.org/10.1111/j.1365-2672.2008.04094.x) PMID: [19187144](https://pubmed.ncbi.nlm.nih.gov/19187144/)
33. Gänzle M, Ehmann M, Hammes W. Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of sourdough fermentation. *Appl Environ Microbiol.* 1998; 64: 2616–2623. PMID: [9647838](https://pubmed.ncbi.nlm.nih.gov/9647838/)
34. Stolz P, Böcker G. Technology, properties and applications of sourdough products. *Adv Food Sci.* 1996; 18: 234–236.
35. Kurtzman C, Robnett C, Ward J, Brayton C, Gorelick P, Walsh T. Multigene phylogenetic analysis of pathogenic candida species in the *Kazachstania* (*Arxiozyma*) *telluris* complex and description of their ascospore states as *Kazachstania bovina* sp. nov., *K. heterogenica* sp. nov., *K. pintolopesii* sp. nov., and *K. slooffiae*. *J Clin Microbiol.* 2005; 43: 101–111. PMID: [15634957](https://pubmed.ncbi.nlm.nih.gov/15634957/)
36. Mihhalevski A, Sarand I, Viiard E, Salumets A, Paalme T. Growth characterization of individual rye sourdough bacteria by isothermal microcalorimetry. *J Appl Microbiol.* 2011; 110: 529–540. doi: [10.1111/j.1365-2672.2010.04904.x](https://doi.org/10.1111/j.1365-2672.2010.04904.x) PMID: [21143710](https://pubmed.ncbi.nlm.nih.gov/21143710/)
37. Vera A, Rigobello V, Demarigny Y. Comparative study of culture media used for sourdough lactobacilli. *Food Microbiol.* 2009; 26: 728–733. doi: [10.1016/j.fm.2009.07.010](https://doi.org/10.1016/j.fm.2009.07.010) PMID: [19747606](https://pubmed.ncbi.nlm.nih.gov/19747606/)
38. Ercolini D. High-Throughput Sequencing and Metagenomics: Moving Forward in the Culture-Independent Analysis of Food Microbial Ecology. *Appl Environ Microbiol.* 2013; 79: 3148–3155. doi: [10.1128/AEM.00256-13](https://doi.org/10.1128/AEM.00256-13) PMID: [23475615](https://pubmed.ncbi.nlm.nih.gov/23475615/)