



## Evaluation of the variations in chemical and microbiological properties of the sourdoughs produced with selected lactic acid bacteria strains during fermentation

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### ABSTRACT

This research aimed to analyze variations in chemical properties, microbiological characteristics and generated volatile organic compounds (VOCs) profile during sourdough fermentation. Sourdoughs were collected from different cities in Turkey at two different times and lactic acid bacteria (LAB) in the samples were identified with culture-independent and culture-dependent molecular methods. According to culture-dependent methodology, thirteen LAB species were identified. *Lactobacillus* spp. were identified as the major group according to MiSeq Illumina analysis. Technological potential of commonly isolated LAB species was evaluated. Due to high frequency of isolation, *Fructilactobacillus sanfranciscensis* and *Lactiplantibacillus plantarum* strains were better investigated for their technological traits useful in sourdough production. Experimental sourdoughs were produced with mono- and dual-culture of the selected strains and chemical properties and microbiological characteristics, as well as VOCs profile of the sourdoughs, were subjected to multivariate analysis which showed the relevance of added starter, in terms of acidification and VOCs profile.

### 1. Introduction

The use of sourdough in bread production is the primary form of bread leavening. In recent years, there is a growing interest of scientists, consumers and industries for sourdough compared to other leavening agents due to the enhanced nutritional quality, improved texture and functional properties, prolonged shelf life, pronounced flavor, and natural production, i.e., without using any additive as a result of its unique and complex microbial composition (Arora et al., 2021). Traditional sourdough bread is mostly produced in retail and artisan bakeries but industrial-scale production has been increasing.

Sourdough is a mixture of flour and water that is fermented by a microbial ecosystem mainly including lactic acid bacteria (LAB) and yeasts (De Vuyst et al., 2021). Sourdough microbiota determines bread

characteristics in terms of acid production, leavening and aroma (Gänzle & Zheng, 2019; Moroni et al., 2009). The leavening and acidifying capacities of the dough are optimized by consecutive refreshments which are also known as back-slopping (Corsetti, 2013; Corsetti & Settanni, 2007). Back-slopping is the addition of flour and water mixture inoculum that is fermented at a defined temperature for a certain time to begin the fermentation of a new mixture of flour and water (Corsetti, 2013). Type I sourdough production process is conducted daily by back-slopping that keeps LAB and yeasts in a metabolically active state (Arora et al., 2021; De Vuyst et al., 2021). Biochemical transformations that occur during sourdough fermentation as a result of the metabolic activities of LAB and also yeasts, improve the functional properties of the dough and final bread (Boyaci Gunduz & Erten, 2019; Gobbetti et al., 2019). The metabolic activities of the sourdough microorganisms result

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in variations in the chemical and microbiological characteristics that affect sourdough performance with improved sensorial and rheological attributes (Arora et al., 2021). LAB are mainly responsible for sourdough fermentation and can originate from raw materials mainly cereal grains or flours and applied technology (Alfonzo et al., 2017; De Vuyst et al., 2021). The microbiota of sourdough is influenced by the sourdough type, dough hydration level, back-slopping time, environment, flour type, fermentation conditions and processing parameters and also, geographical origin (De Vuyst et al., 2017; Lau et al., 2021). Sourdoughs usually include associations of different heterofermentative and homofermentative LAB strains and especially LAB group, formerly reported as *Lactobacillus* spp. and recently renamed by Zheng et al. (2020), occur in sourdough ecosystems (Arora et al., 2021). Different LAB communities in stabilized sourdoughs at different environments have been identified worldwide. LAB species isolated from Turkish sourdoughs by phenotypic and genotypic methods are given in Supplementary Table S1 (Dertli et al., 2016; Gül et al., 2005; Mentis et al., 2004; Sevgili et al., 2021; Simsek et al., 2006; Yagmur et al., 2016). Identification of sourdough microbiota is very important to understand microbial ecology for optimizing fermentation conditions (Lau et al., 2021) and designing starter culture combinations for the production of sourdough bread at the industrial level. This is due to the increasing demand for sourdough because it provides the final products with a natural image. Therefore, industrial production with starter culture addition might ensure repeatability and consistency of sourdough bread production and to achieve a controlled fermentation at industrial scale, starter culture combinations should be designed. A successful starter culture design could be achieved by selection of the strains already adapted to the specific sourdough environment conditions.

The objective of the present study was to isolate the predominant LAB microbiota of sourdough samples collected from some parts of Turkey and to select LAB strains showing the most relevant performances to act as starter culture for experimental sourdough fermentation to provide the final products with enhanced properties and to be applied at industrial level. Furthermore, Next Generation Sequences analysis was applied to deeply investigate the microbiota composition and diversity among the different Turkish bakeries.

**Hypotheses:** *Starter culture addition ensures faster acidification during sourdough fermentation. Strains isolated from the specific sourdough environments show good technological traits to be used as starter culture in sourdough fermentations.*

## 2. Materials and methods

### 2.1. Sample collections

Totally eight sourdough samples were collected from three commercial bakeries located in different cities, Mersin (36°46'26.7"N 34°34'22.9"E), Antalya (36°53'08.6"N 30°43'40.8"E) and Ankara (39°56'57.9"N 32°46'05.9"E), in Turkey. Bakeries were selected based on their sourdough production without baker's yeast addition. Samples were taken at two different times between April 2016 and February 2017. Three whole-meal wheat sourdoughs, a total of 6, and a rye sourdough sample, a total of 2 samples, were taken from the bakeries at two different times. A code that consists of letter and sampling time was given to each collected sample without expressing the bakery names, with SD denoting sourdough, and a randomly chosen letter, and 1 or 2 indicating the first or second sampling. Collected samples were whole-meal wheat sourdoughs except for R coded sourdough which was rye sourdough. All samples were collected in duplicate before the daily refreshment step. Samples were taken into sterile jars and kept at 4 °C until analysis.

### 2.2. MiSeq library preparation

The amplification target was a 464-nucleotide sequence of the bacterial V3-V4 region of the 16S rRNA gene (*Escherichia coli* positions 341 to 805) (Claesson et al., 2010). Sample pooling and differentiation were obtained through the attachment of unique barcodes before forwarding primers. The amplicons were cleaned using the Agencourt AMPure kit (Beckman Coulter, Milano, Italy) and DNA concentration was determined by the Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA). Library preparation and pair-end sequencing were carried out at the Genomic Platform-Fondazione Edmund Mach (San Michele all'Adige, Trento, Italy) applying Illumina MiSeq technology (Illumina, San Diego, CA) as reported previously (Boyaci Gunduz et al., 2020).

### 2.3. Illumina data analysis and sequences identification by QIIME2

After demultiplexing by idemp (<https://github.com/yhwu/idemp/blob/master/idemp.cpp>), raw paired-end FASTQ files were imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2). Sequences were quality-filtered, trimmed, denoised, and merged using DADA2. Illumina data analysis and sequences identification were done as reported previously (Boyaci Gunduz et al., 2020). For taxonomy tables, a pre-trained Naive Bayes classifier using the Greengenes 13.8 99% Operational Taxonomic Units (OTUs) database (<https://greengenes.secondgenome.com/>) was applied to paired-end sequence reads. The resulting data were deposited in the NCBI Sequence Read Archive (SRA) under Ac. PRJNA638506.

### 2.4. Isolation of presumptive LAB

For isolation of presumptive LAB, three media including modified de Man Rogosa Sharpe (mMRS) (Merck) agar, modified glucose M17 (gM17) (Merck) agar and sourdough bacteria agar (SDB) (Settanni et al., 2011) prepared with 10% fresh yeast extract solution (v/v), 2% maltose (w/v), 0.3% yeast extract (w/v), 0.6% pancreatic digest of casein (w/v), 0.03% Tween 80 (v/v) and 1.5% agar (w/v) were used. Plates were incubated anaerobically at 30 °C for 48–72 h (h). The colonies were grouped according to their shape, color, edge and size, and at least 10–15 colonies/plate were randomly picked and streaked onto a single agar plate containing appropriate agar media for isolation by the plate-streaking technique. Streaked plates were incubated at 30 °C for 48 h anaerobically. When all of the colonies on the plate had the same general appearance, a colony was picked and subsequently transferred into the corresponding broth media and incubated at 30 °C for 48 h. Each colony that had a different appearance on a plate was streaked again onto a separate plate until a pure culture was obtained. Then, isolated presumptive LAB (Gram-positive, catalase-negative) colonies were transferred into mMRS broth (Merck) containing 40% (v/v) sterile glycerol (Merck) solution and stored at – 25 °C until identification.

### 2.5. Randomly amplified polymorphic DNA (RAPD-PCR) analysis

Stored LAB isolates were activated overnight at 30 °C in MRS broth media and genomic DNA was extracted using the InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Differentiation of the potential LAB isolates was performed by RAPD-PCR analysis using the M13 primer (5'-GAGGGTGGCGGT TCT-3') according to the protocol reported previously (Boyaci Gunduz et al., 2020). RAPD-PCR profiles were analyzed through the software package Gelcompare II Version 6.5 (Applied Maths, Sin-Martens-Latem, Belgium). Then, one or two LAB isolates of each cluster were chosen to be identified by 16S rRNA gene sequencing.

### 2.6. Molecular identification of LAB by 16S rRNA gene sequence analysis

LAB with different RAPD-PCR profiles were subjected to 16S rRNA

gene sequence analyses for molecular identification. PCR amplification was performed using primers fD1(5'-AGAGTTTGATCCTGGCTCAG-3', Thermo Scientific) and rD1(5'-AAGGAGGTGATCCAGCC-3', Thermo Scientific) as reported previously (Boyaci Gunduz et al., 2020). PCR amplicons were sequenced at BM Laboratory Systems (Ankara, Turkey). ABI chromatograms of the sequences were evaluated, multiple alignments were performed using ClustalW Multiple alignment (Bioedit version 7.0.9) and then the resulting sequences were compared with nucleotide sequences deposited at the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). LAB species identity was determined based on the similarity to the reference sequences of 16S rRNA gene sequences with a threshold of 98%.

## 2.7. Exploration of some technological properties of selected LAB isolates to be used as starter culture in sourdough fermentation

The strains most frequently isolated were examined for their technological potential to be used as starter cultures in sourdough fermentations. Technological analyses were conducted in duplicate.

### 2.7.1. Acidification activity

The acidification activities of the selected strains were investigated in sterile flour extract (SFE) broth according to the method described previously (Alfonzo et al., 2013). Selected LAB cultures were grown overnight at 30 °C in MRS broth and harvested by centrifugation at 13,300 rpm for 3 min (Thermo Scientific MicroCL 17, Germany), washed with sterile Ringer's solution and suspended again in the same solution to an optical density (OD) at 600 nm ( $OD_{600}$ ) of 1.00 (Shimadzu UV-1700, Japan) to standardize bacterial inocula. Twenty mL of SFE were inoculated with 1% (v/v) of the cell suspension at a final cell density of ca.  $10^6$  CFU/mL. Incubation of the tubes was conducted at 30 °C and pH was monitored for the first 8 h of incubation at 2-h intervals and then at 24, 48, 72 h and 7 d after inoculation. Control was the uninoculated SFE.

In addition, the strains were also analyzed for their ability to produce lactic acid. For that purpose, acidified SFE (aSFE) samples after 8 h of fermentation were analyzed through HPLC system according to the procedure explained in section 2.8.3.

### 2.7.2. Antimicrobial activity against some selected pathogens

Selected strains were evaluated for their antimicrobial activity using the dual culture overlay technique against *Bacillus subtilis*, *Bacillus licheniformis*, *Escherichia coli*, *Penicillium expansum* and *Penicillium digitatum*. The indicator strains, *Bacillus subtilis* and *Bacillus licheniformis* were grown in Nutrient broth at 37 °C, *Escherichia coli* in Brain Heart Infusion broth at 37 °C and the molds *Penicillium expansum* and *Penicillium digitatum* in Malt Extract broth at 28 °C until reaching  $OD_{600} = 1.0$ . The plates were incubated at the optimal growth temperature and time for the indicator strains and the detectable clear zone around the colonies of the producer strain was scored as positive inhibition.

### 2.7.3. Growth characteristics under different conditions

The selected strains ( $OD_{600} = 1$ ) were inoculated into mMRS broth (1%) to evaluate growth at different temperatures (15, 28, 37 and 45 °C), pH (3.5, 4.5 and 6.5) and salt (4, 6 and 8%) conditions. Each condition was tested separately. The abilities of the strains to ferment various carbohydrates were performed with different sugars including D (+) glucose monohydrate (Sigma-Aldrich), D (-) fructose (Merck), D (+) galactose (Fluka), lactose monohydrate (Merck), sucrose (Merck), maltose monohydrate (Merck), L (+) rhamnose monohydrate, raffinose (Difco), D (-) mannitol (Merck), D (+) mannose (Fluka), D (-) arabinose (Fluka) and D (+) xylose (Sigma Aldrich). For that purpose, each filter-sterilized sugar solution (1%, w/v) was added separately to the tubes including MRS broth prepared without meat extract and glucose. Then each strain ( $OD_{600} = 1$ ) was inoculated individually into each tube. Chlorophenol red (0.004%, w/v) was used as the indicator. The control

broth was prepared without any sugar.

### 2.7.4. Enzyme profile

The enzyme profiles of the selected strains were detected by API ZYM enzyme (Biomérieux, France) testing system according to the manufacturer's instructions using ZYM A and ZYM B reagents.

## 2.8. Production of experimental sourdoughs and evaluation of the chemical and microbiological properties

### 2.8.1. Experimental sourdough production with selected strains

Among mostly isolated LAB species, two strains were characterized for use in sourdough fermentations. Sourdough production under laboratory conditions was performed according to the traditional (sourdough Type I) protocol as shown in Supplementary Figure S1. Selected strains ( $OD_{600} = 1$ ) were inoculated at a concentration of 1% (v/v) into the flour and water mixture at the beginning of the fermentation. A control sourdough was produced without using a starter culture. Sourdough fermentations were conducted in duplicate at 28 °C for 3 d with daily refreshment.

Total titratable acidity (TTA) and pH measurements were conducted for all samples at 4, 8 h and 12 h of the fermentation and every 24 h until the last refreshment of the sourdough production. Plate counting, carbohydrate, organic acid and ethanol analyses were performed on the sourdoughs before the daily back-slopping step. In addition, the volatile organic compounds (VOCs) profile of each sourdough were examined at the end of fermentation by SPME-GC-MS. Samples were analyzed in duplicate.

### 2.8.2. Investigation of microbiological parameters

Cell suspensions of experimental sourdough samples were analyzed by plate count for the enumeration of the following microbial groups: mesophilic LAB on MRS, yeasts and molds on yeast peptone dextrose (YPD), total mesophilic aerobic microorganisms on Plate Count Agar (PCA) and presumptive coliform group bacteria in Lauryl Sulfate Tryptose broth (Merck) as reported previously (Boyaci Gunduz et al., 2020). 10 g of dough sample was suspended with 90 mL of sterile 0.85% (wt/vol) NaCl (Merck) solution in sterile stomacher bags and homogenized for 3 min at the maximum speed using a bag mixer (Interscience, model 400P, France). 10-fold dilution series of the sourdough samples were prepared by transferring a volume of 1 mL into test tubes containing 9 mL of NaCl solution. Aliquots of the decimal dilutions were spread onto modified de Man Rogosa Sharpe (mMRS) (Merck) [including 1% maltose (w/v) and 5% fresh yeast extract solution (v/v)] agar media to allow the growth of LAB. Incubation was performed anaerobically using the Anaerocoult A packs (Merck 1.13829) in sealed jars at 30 °C for 48–72 h. YPD plates were incubated aerobically for yeast and mold growth at 28 °C for 48 h and 7 d, respectively. Total mesophilic aerobic microorganisms were counted on plate count agar (PCA) incubated aerobically at 30 °C for 3 d. Selected media were supplemented with different sterile filtered (Millex-GS, 0.22 µm filter) antibiotics according to the target organism. For that purpose, cycloheximide (0.1 g/L, Sigma), oxytetracycline (0.1 g/L, Sigma) and sodium propionate (2 g/L, Sigma-Aldrich) antibiotics were used to suppress yeast, bacteria and mold growth, respectively. Colonies were counted and results were expressed as log CFU/g.

### 2.8.3. Investigation of chemical characteristics

Ten g of sample was homogenized with 90 mL of distilled water and the pH was measured using a digital glass pH meter (Mettler Toledo, SevenCompact™ pH Ion S220, Switzerland). For determination of TTA, sample homogenate was titrated with 0.1 N NaOH to a final pH of 8.5. TTA was expressed as the amount (mL) of 0.1 M NaOH needed to achieve the pH of 8.5.

Maltose, sucrose, glucose, fructose, ethanol, lactic and acetic acids were determined in the extracts through an HPLC system consisting of a

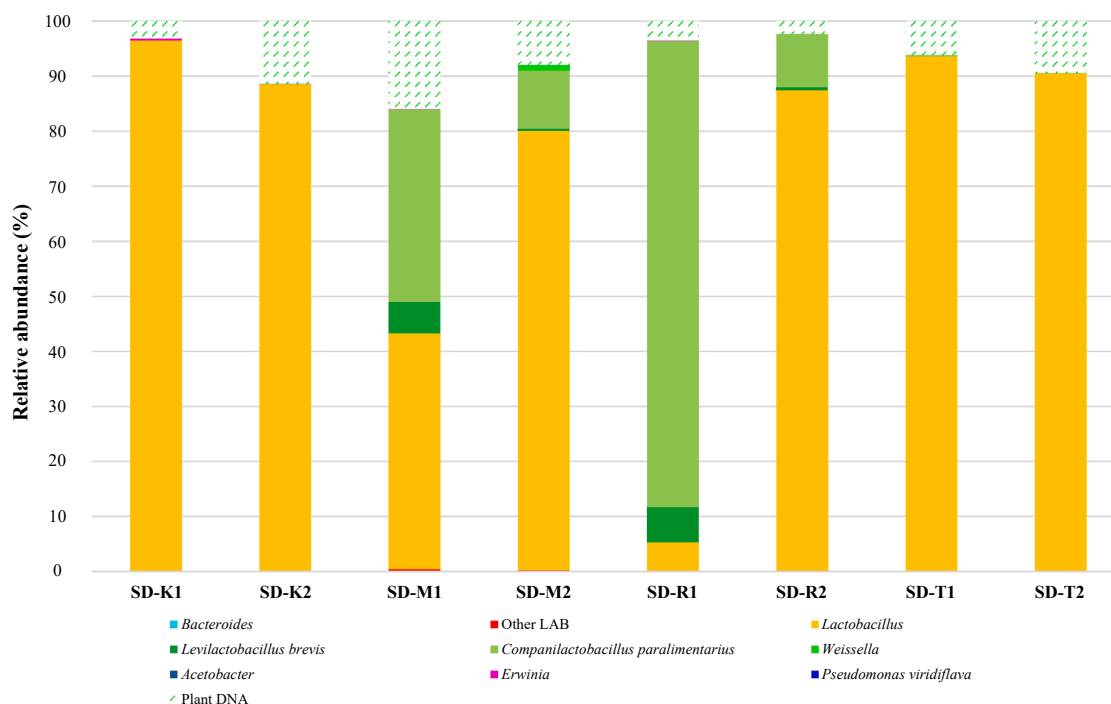


Fig. 1. Relative abundances (%) of bacterial taxa identified by MiSeq Illumina in sourdough samples. Abbreviations: SD, sourdough; R, rye; K, M, and T, whole-meal wheat sourdoughs collected from three different bakeries; 1 and 2 indicate first and second sampling.

refractive index detector (RID-10A) for sugar and ethanol analyses and a UV/Vis detector (SPD-20A) monitored at 210 nm for organic acid analyses as reported previously (Boyaci-Gunduz & Erten, 2020). Extraction was done according to the method of Paramithiotis et al. (2006). An Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) was used for chromatographic separation. HPLC analyses were carried out in duplicate.

#### 2.8.4. Investigation of volatile organic compounds

VOCs generation was determined according to the method of Settanni et al. (2013) with some modifications. The solid phase micro extraction (SPME) technique was used with the SPME fiber (85 μm Carboxen/PDMS) and GC/MS system (Agilent 7000 Series Triple Quad) equipped with an HP – 5MS capillary column (30 m, 0.250 mm i.d., film thickness 0.25 μm, %5 phenyl methyl poly siloxane). Ionizing energy was 70 eV and MS was at the full-scan mode with a scan range of 50–600 *m/z*. The carrier gas was helium at a constant flow rate of 1.0 mL/min. Results were evaluated according to the National Institute of Standards and Technology (NIST 14L) reference library and VOCs were expressed as relative peak areas (peak area of each compound/total area\*100).

#### 2.9. Evaluation of the properties by statistical univariate and explorative multivariate analysis

Results of the analysis were subjected to one-way analysis of variance (ANOVA) and multiple comparisons of means by *post hoc* Tukey's procedure by Statistical Package for Social Science 20.0 software (International Business Machines Corporation). The dissimilarity index was calculated using Darwin (6.0.15) software package. Multivariate statistical analyses were carried out using XLSTAT 2018 software (Addinsoft) for Microsoft Excel®.

### 3. Results and discussion

#### 3.1. Illumina data analysis of the microbiota in the collected sourdough samples

Sequences obtained from Illumina Sequencing were processed using QIIME2 software. The distribution of the relative abundances (%) of bacterial genera identified by MiSeq Illumina in the samples is shown in Fig. 1. The major part of OTUs belonged to the group of LAB formerly reported as *Lactobacillus* spp. and was recently renamed by Zheng et al. (2020) reaching a maximum relative abundance of 96.48% in sample SD-K1. The samples SD-M1, SD-M2, SD-R1 and SD-R2 were also characterized by the clear presence of *Levilactobacillus brevis* (formerly *Lactobacillus brevis*) and *Companilactobacillus (C.) paralimentarius* (formerly *Lactobacillus paralimentarius*). In particular, *Levilactobacillus brevis* accounted for 84.69% of relative abundance in sample SD-R1. A very low percentage of *C. paralimentarius* was also registered in samples SD-T1 and SD-T2 (0.31 and 0.13%, respectively). The presence of *Weissella* spp. was only observed in sample SD-M2 with a relative abundance of 0.98%. Except for very low relative abundances of *Pseudomonas* spp. (0.14%) in sample SD-M1 all samples were characterized by the presence of species with useful roles in sourdough propagation. Even though 16S-based metagenomics could optimally resolve taxonomies only to the genus level, it sometimes contains sufficient information to identify at the species level. This approach was successfully applied by Michel et al. (2016) to evaluate the dominance of *F. sanfranciscensis* in 13 of 16 organic French sourdoughs analyzed through MiSeq Illumina technology. This technique is particularly useful to reveal the dominance of LAB species in back-slopped wheat and rye sourdoughs even after only one day of fermentation (Alfonzo et al., 2017; Bessmeltseva et al., 2014; Celano et al., 2016; Ercolini et al., 2013). Thus, metagenomics is important to avoid the underestimation of sourdough-characteristic LAB species (Weckx et al., 2019).



**Table 1**  
Distribution of LAB in sourdough samples.

Species	Samples							
	M1	M2	T1	T2	K1	K2	R1	R2
<i>Fructilactobacillus sanfranciscensis</i>		2/	1/	10/	18/	4/		2/
		12	7	23	22	14		6
<i>Lactiplantibacillus plantarum</i>	5/	2/		2/	1/	9/	1/	
	8	12		23	22	14	5	
<i>Companilactobacillus paralimentarius</i>	1/	4/	3/	2/			2/	3/
	8	12	7	23			5	6
<i>Lacticaeibacillus paracasei</i>			2/	6/				
			7	23				
<i>Weissella confusa</i>		4/						
		12						
<i>Levilactobacillus brevis</i>							2/	1/
							5	6
<i>Lactiplantibacillus pentosus</i>					2/			
					22			
<i>Leuconostoc citreum</i>				2/				
				23				
<i>Lactiplantibacillus paraplanctarum</i>	2/							
	8							
<i>Lactobacillus acidophilus</i>			1/					
			7					
<i>Enterococcus faecium</i>				1/				
				23				
<i>Pediococcus inopinatus</i>					1/			
					22			
<i>Levilactobacillus parabrevis</i>						1/		
						14		
Total LAB isolates	8	12	7	23	22	14	5	6

### 3.2. Biodiversity of the LAB in the sourdough samples

All presumptive LAB cultures were subjected to microscopic inspection and putative LAB cultures (Gram-positive and catalase-negative) were subjected to DNA extraction. All isolates were subjected to RAPD analysis to differentiate the strains constituting the microbiota of the sourdoughs object of investigation. According to the calculated genetic distance matrix with the UPGMA method, the strains characterized by a genetic distance of 0.4 or more were chosen for sequence analysis. Based on the 16S rRNA sequence analysis, a total of 59 strains representing 141 isolates were confirmed to be members of the LAB group with a sequence length of more than 1250 bp. Among them, 38 strains (1400 bp  $\leq$ ) representing 97 isolates were identified at the species level (similarity  $\geq$  98%) as shown in [Supplementary Table S2](#). Other strains were identified at the genus (similarity  $\geq$  94%) or family (similarity  $\geq$  86%) level as shown in [Supplementary Table S3](#).

RAPD patterns were analyzed separately for rods and cocci resulting in two dendrograms ([Supplementary Figure S2](#)). Cocci constituted a minority of the sourdough LAB community, while rods were confirmed to be the main LAB of mature Turkish sourdoughs. The group of LAB rods was particularly diverse; basically, the main clusters included *Fructilactobacillus (F.) sanfranciscensis* (formerly *Lactobacillus sanfranciscensis*), *Lactiplantibacillus (L.) plantarum* (formerly *Lactobacillus plantarum*) and *C. paralimentarius*. The frequency of the LAB species indicated a consistent presence of lactobacilli, in particular; *F. sanfranciscensis* (38.14%), *L. plantarum* (20.62%) and *C. paralimentarius* (15.46%) as shown in [Supplementary Table S4](#). The distribution of LAB species in sourdough samples is shown in [Table 1](#). *Lactobacillus* spp. is largely the most abundant in stable sourdough ecosystems ([Arora et al., 2021](#)). Other less predominant LAB species, including members of the genera *Weissella*, *Pediococcus*, *Leuconostoc*, *Lactococcus*, *Enterococcus* and *Streptococcus* can be found in sourdoughs but at lower levels than *Lactobacillus* spp. ([Corsetti & Settanni, 2007](#)). In the present study, MiSeq Illumina confirmed that the recently renamed *Lactobacillus* spp. constituted the major LAB group. The apparent discrepancy between *Lactobacillus* species identified by Illumina and identified following isolation could be imputable to the fact that through

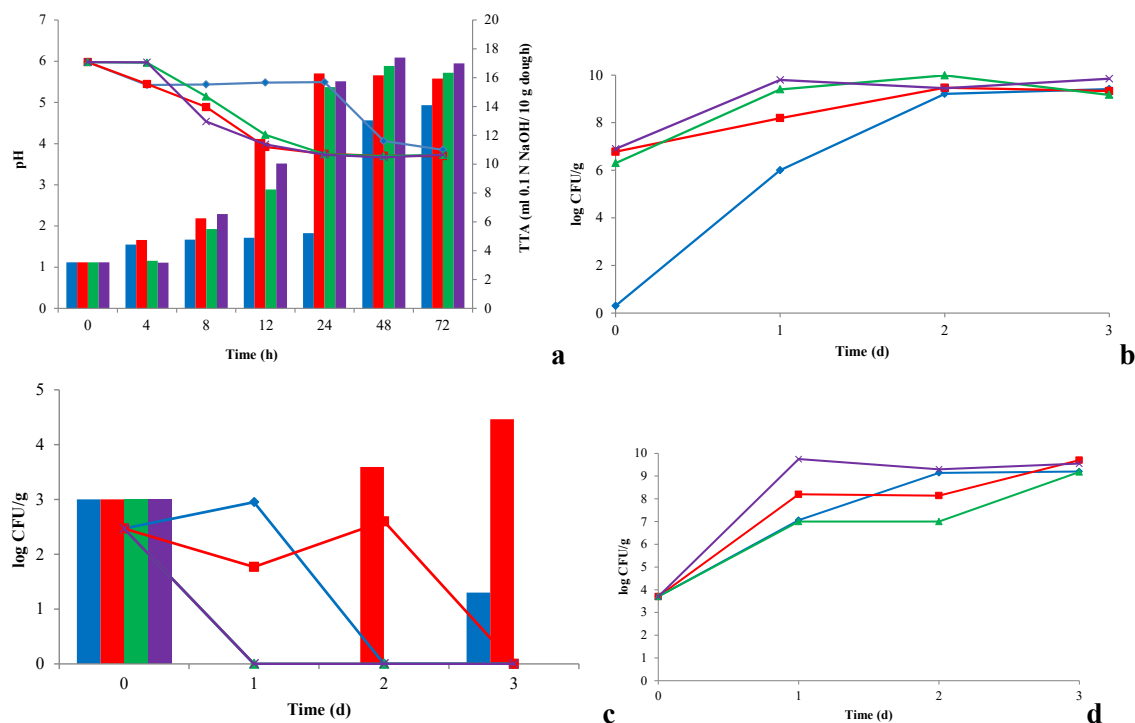
Illumina LAB remained unassigned or their abundances were below 0.1% or their DNAs were rendered inaccessible ([Gaglio et al., 2020](#)). In our study, microbial patterns of the sourdoughs collected from different bakeries differed between each other and around three-quarters of the identified strains belonged to the genus *Lactobacillus* spp. according to the genus and species-level identification. The predominant LAB species, *F. sanfranciscensis*, mainly dominated the sourdough ecosystem and *L. plantarum* and *C. paralimentarius* were identified as the sub-dominant species. *F. sanfranciscensis* is a key bacterium in traditional Type I sourdoughs produced with continuous propagation by back-slopping procedures since it is perfectly adapted to the sourdough surroundings ([Rogalski et al., 2021a](#)). As reported previously, this species is well known in natural sourdough habitats of the artisan and industrial bakeries ([Landis et al., 2021](#); [Lhomme et al., 2016](#); [Gänzle & Zheng, 2019](#)). *L. plantarum* and *C. paralimentarius* has been frequently reported in many sourdoughs as the sourdough-specific LAB species that predominate the sourdoughs ([Arora et al., 2021](#); [Minervini et al., 2018](#); [Taccari et al., 2016](#)).

Other minor species identified in the present study include *Lacticaeibacillus paracasei* (formerly *Lactobacillus paracasei*), *Weissella confusa*, *Levilactobacillus brevis*, *Lactiplantibacillus pentosus* (formerly *Lactobacillus pentosus*), *Leuconostoc citreum*, *Lactiplantibacillus paraplanctarum* (formerly *Lactobacillus paraplanctarum*), *Lactobacillus acidophilus*, *Enterococcus faecium*, *Pediococcus inopinatus* and *Levilactobacillus parabrevis* (formerly *Lactobacillus parabrevis*).

A large diversity of lactobacilli grow in sourdoughs ([Arora et al., 2021](#); [Gänzle & Zheng, 2019](#)). In the present study, *Lactobacillus* spp. dominate the sourdoughs, but, species distribution and dominant microbiota varied among the collected sourdough samples indicating the influence of the different environmental conditions on the sourdough ecosystem. According to [De Vuyst et al. \(2017\)](#), the sourdough ecosystem can contain a simple microbiota characterized by *L. plantarum* or *F. sanfranciscensis* or a restricted LAB species diversity or with a complex microbial consortium including different LAB species generally less than three species. Flour type and its quality and the process parameters such as fermentation temperature, pH and pH evolution, water activity, dough yield, oxygen tension, back-slopping procedure and fermentation duration directly determine the dynamics and outcome of back-slopped sourdough fermentation processes ([De Vuyst et al., 2017](#)). As reported previously, temperature is the main parameter affecting dominant microbiota and microbial activity based on seasonal fluctuations and baking environment conditions and this results in varied physical, chemical and volatile properties of sourdough ([Calvert et al., 2021](#); [Siepmann et al., 2019](#)). In the present study, LAB species varied in the samples collected from different locations and even in the samples collected from the same location at two different times. This result showed that the typical sourdough microbiota of a mature sourdough produced at a location could be changed. A recent comprehensive study was conducted to investigate the diversity and function of sourdough starter microbiomes and reported that geographical location has little influence on the microbial diversity of sourdough since geographic location did not correlate with the diversity of the sourdough starter cultures ([Landis et al., 2021](#)).

### 3.3. Evaluation of the technological attributes of selected LAB

Each sourdough starter contains unique and complex microorganisms ([Calvert et al., 2021](#)). Therefore, variations in physical, chemical, microbiological and sensorial properties were observed during sourdough fermentation which result in sourdough bread with different characteristics. Therefore, the identification of sourdough microbiota and variations depending on the different strains should be evaluated to design starter culture combinations for the production of sourdough bread at the industrial level. In this study, members of the most frequently isolated species, *F. sanfranciscensis*, *L. plantarum* and *C. paralimentarius*, were explored for their fermentative potential.



**Fig. 2.** a Kinetics of acidification during fermentation. Histogram graph: TTA. Line graph: pH. Colors: Blue, control sourdough; red, experimental sourdoughs inoculated with *L. plantarum* XL23; green, experimental sourdoughs inoculated with *F. sanfranciscensis* RL976; purple, experimental sourdoughs inoculated with inoculated with *L. plantarum* XL23 + *F. sanfranciscensis* RL976. 2b. Microbial loads of rod LAB during fermentation. Colors: Blue, control sourdough; red, experimental sourdoughs inoculated with *L. plantarum* XL23; green, experimental sourdoughs inoculated with *F. sanfranciscensis* RL976; purple, experimental sourdoughs inoculated with inoculated with *L. plantarum* XL23 + *F. sanfranciscensis* RL976. 2c. Microbial loads of yeasts during fermentation. Histogram graph: yeasts. Line graph: molds. Colors: Blue, control sourdough; red, experimental sourdoughs inoculated with *L. plantarum* XL23; green, experimental sourdoughs inoculated with *F. sanfranciscensis* RL976; purple, experimental sourdoughs inoculated with inoculated with *L. plantarum* XL23 + *F. sanfranciscensis* RL976. 2d. Microbial loads of total mesophilic aerobic microorganisms during fermentation. Colors: Blue, control sourdough; red, experimental sourdoughs inoculated with *L. plantarum* XL23; green, experimental sourdoughs inoculated with *F. sanfranciscensis* RL976; purple, experimental sourdoughs inoculated with inoculated with *L. plantarum* XL23 + *F. sanfranciscensis* RL976.

Experimental sourdough production with starter culture was conducted with mono- and dual-culture. *F. sanfranciscensis* strains were chosen for the investigation of their technological potential to be used as starter in sourdough fermentation. For the selection of other strain, the properties of *L. plantarum* and *C. paralimentarius* species were investigated and compared.

Firstly, selected LAB strains were evaluated for their acidification rates. Acidification and growth rates are the most examined properties to conduct sourdough fermentation in a shorter time which is important, particularly at the industrial scale (Arora et al., 2021). For this purpose, the strains were subjected to the acidification test and the results are shown in Supplementary Table S5. At 24 h, almost all of the strains acidified the medium to below pH 4.0. After 3 days (d), the lowest pH values were measured in the SFEs inoculated with *L. plantarum* species. *L. plantarum* XL23 showed the lowest pH value at the 7th d. Among *F. sanfranciscensis* strains, RL976 exhibited the lowest acidity values during fermentation of SFE. In addition, after 8 h of fermentation, the lactic acid content of acidified SFE was found to be in the range of 0.57–1.15 mg/g for all species. The highest lactic acid content was detected in the aSFE inoculated with *L. plantarum* XL23. The lactic acid content of acidified SFEs inoculated with different species was in the range of 0.67–0.72 mg/g, 0.59–1.15 mg/g and 0.57–1.02 mg/g for *C. paralimentarius*, *L. plantarum* and *F. sanfranciscensis*, respectively. These values are comparable with those reported in the literature; Alfonzo et al. (2013) analyzed the ability of the organic acid production in SFE broths inoculated with different *Lactobacillus* spp., *Leuconostoc* spp. and *Weissella* spp. and, after 8 h of fermentation, the content of

lactic acid was in the range of 0.33–0.59 mg/g, while Ventimiglia et al. (2015) reported a concentration from 0.42 to 0.81 mg/g when SFE broths were fermented by *L. plantarum* strains. The works performed inoculating the single strains in flour rather than SFE broths report higher concentrations of lactic acid. At the same fermentation duration (8 h), Settanni et al. (2013) reported that the lactic acid produced by different LAB strains in sourdoughs processed with non-sterile flour ranged between 1.36 and 6.47 mg/g, while Ventimiglia et al. (2015) reported a lactic acid content of 1.48–4.19 mg/g for the sourdoughs started with *L. plantarum* strains. The differences among the concentrations of lactic acid of SFEs and sourdoughs could be due to the first matrices containing lower amounts of nutrients as being prepared from 20% (w/v) flour (Alfonzo et al., 2016).

In sourdough fermentation, rapid acidification is an important technological characteristic of LAB (Corsetti & Settanni, 2007). According to the acidification activity results, *C. paralimentarius* exhibited less acidification compared to the *L. plantarum* species. Since fast acidification is an important characteristic for a sourdough starter culture to be applied at industrial scale level, *C. paralimentarius* strains of the present study were eliminated for further evaluation. In addition, it has been previously reported that *L. plantarum* could be an ideal starter culture for the Type I sourdoughs (Minervini et al., 2010). The acid production capacity of *L. plantarum* XL23 was very high among *L. plantarum* species. After 8 h, pH of the aSFE was the lowest in *L. plantarum* XL23. Also according to the final pH values on the 7th day, the lowest pH was determined in the same strain among *L. plantarum* strains. After 8 h, pH values were close to each other among

*F. sanfranciscensis* strains. On the other hand, the lowest pH values at the 7th d were determined in the aSFE inoculated with *F. sanfranciscensis* RL976 compared to other *F. sanfranciscensis* species. Therefore, *L. plantarum* XL23 and *F. sanfranciscensis* RL976 were chosen for their acidification capacity in experimental sourdough production.

According to the results of antimicrobial analysis, *L. plantarum* XL23 showed inhibitory activity against *Bacillus subtilis*, *Bacillus lincheniformis*, *Escherichia coli* and *Penicillium expansum*. *F. sanfranciscensis* RL976 showed inhibitory activity only against *B. subtilis*. It was reported that *L. plantarum* showed a very and inhibited many mold species including *Penicillium* spp. (Lavermicocca et al., 2000). Corsetti et al. (1996) investigated the *Lactobacillus* spp. isolated from sourdoughs and reported all the strains were inhibitory to *Bacillus subtilis* and among the strains *F. sanfranciscensis* and *L. plantarum* strains had the largest inhibitory spectrum. However, the inhibitory spectrum among strains of the same species varied. Similarly, a recent study was conducted to screen antimicrobial activities of LAB strains isolated from sourdoughs and *L. plantarum* and *F. sanfranciscensis* strains showed inhibitory effects against the indicator strains. *L. plantarum* exhibited the highest antifungal potential and strain-dependent variations in antimicrobial activities were observed against the indicator strains (Fraberger et al., 2020).

The growth of selected strains under different temperatures, pHs and salt concentrations were investigated. Both of the strains were able to grow at 15 °C, 28 °C and 37 °C. None of the strains displayed any growth at 45 °C. *L. plantarum* XL23 strain was able to grow at all analyzed salt concentrations and pH conditions. On the other hand, *F. sanfranciscensis* RL976 strain did not display any growth under two different conditions, pH 3.5 and in the presence of 8% NaCl concentration. Moreover, the abilities of *L. plantarum* XL23 and *F. sanfranciscensis* to ferment different carbohydrates were evaluated according to the color change in the tube since color change from yellow to violet is observed at decreased pH values due to the growth and production of lactic acid. Color change due to the acid production was observed in the tubes containing glucose, fructose, sucrose, maltose, galactose, lactose, mannose and mannitol for both strains. Raffinose was only used by *L. plantarum* XL23. None of the investigated strains used xylose, rhamnose and arabinose as carbohydrate sources. Strain-specific differences could be observed in the growth under different conditions. Similar results were reported by a recent study examining the genome-phenotype-associations in *F. sanfranciscensis* strains through physiological and genomic analyses (Rogalski et al. 2021b). The study reported no growth of *F. sanfranciscensis* strains on pentoses including xylose and arabinose as the sole carbon source possibly due to the absence of or mutations in numerous genes essential for arabinose and xylose metabolism.

In addition, enzyme activities of the selected *F. sanfranciscensis* and *L. plantarum* strains were investigated. The application of starter cultures with enzyme activities for specific compounds could improve the functionality of the final sourdough bread. In the present study, the strains of both species produced enzymes as follows: leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-Bi-phosphohydrolase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase and *N*-acetyl- $\beta$ -glucosaminidase. On the other hand,  $\beta$ -galactosidase activity was only observed for *L. plantarum* XL23 strain.

### 3.4. Production of experimental sourdoughs with selected strains and evaluation of chemical and microbiological properties during fermentation

Based on the technological screening, *L. plantarum* XL23 and *F. sanfranciscensis* RL976 were selected to be used as starter culture for experimental sourdough production. Experimental sourdoughs were produced with mono- and dual-culture of the selected strains and chemical properties and microbiological characteristics, as well as VOCs profile of the sourdoughs were analyzed. The pH and TTA values registered for the experimental sourdoughs are shown in Fig. 2a. The final pH and TTA values of the sourdoughs were in the range of

**Table 2**  
Sugar, organic acid and ethanol contents (g/kg) of experimental sourdoughs.

Compounds	Refreshment (day)	Experimental sourdoughs			
		SD-C	SD-1	SD-2	SD-3
Maltose + sucrose					
0		12.03 <sup>a</sup>	12.03 <sup>a</sup> ± 0.098	12.03 <sup>a</sup> ± 0.098	12.03 <sup>a</sup> ± 0.098
	1	13.25 <sup>a</sup>	11.11 <sup>b</sup> ± 0.09	13.15 <sup>a</sup> ± 1.15	13.92 <sup>a</sup> ± 0.41
		14.48 <sup>a</sup>	9.84 <sup>b</sup> ± 0.68	9.87 <sup>b</sup> ± 0.29	10.07 <sup>b</sup> ± 0.44
		13.34 <sup>a</sup> ± 0.47	9.19 <sup>b</sup> ± 0.89	9.76 <sup>b</sup> ± 0.14	9.87 <sup>b</sup> ± 0.90
Glucose					
0		7.41 <sup>a</sup> ± 0.27	7.41 <sup>a</sup> ± 0.27	7.41 <sup>a</sup> ± 0.27	7.41 <sup>a</sup> ± 0.27
	1	7.08 <sup>ab</sup> ± 1.09	9.64 <sup>b</sup> ± 0.49	8.18 <sup>ab</sup> ± 0.04	6.15 <sup>a</sup> ± 0.41
		10.03 <sup>a</sup> ± 1.03	9.36 <sup>ab</sup> ± 0.12	8.89 <sup>a</sup> ± 0.50	7.81 <sup>b</sup> ± 0.46
		10.51 <sup>c</sup> ± 0.90	8.45 <sup>a</sup> ± 0.28	10.03 <sup>bc</sup> ± 0.50	6.17 <sup>a</sup> ± 0.74
Fructose					
0		6.21 <sup>a</sup> ± 0.66	6.21 <sup>a</sup> ± 0.66	6.21 <sup>a</sup> ± 0.66	6.21 <sup>a</sup> ± 0.66
	1	4.08 <sup>b</sup> ± 0.60	2.36 <sup>a</sup> ± 0.17	4.50 <sup>b</sup> ± 0.11	2.76 <sup>a</sup> ± 0.06
		3.17 <sup>b</sup> ± 0.24	2.31 <sup>a</sup> ± 0.04	3.01 <sup>bc</sup> ± 0.02	2.56 <sup>ab</sup> ± 0.31
		3.80 <sup>b</sup> ± 0.89	2.17 <sup>a</sup> ± 0.25	2.52 <sup>ab</sup> ± 0.44	2.47 <sup>ab</sup> ± 0.19
Ethanol					
0		<LOQ	<LOQ	<LOQ	<LOQ
	1	2.70 <sup>b</sup> ± 0.00	2.44 <sup>a</sup> ± 0.12	2.52 <sup>a</sup> ± 0.02	<LOQ
		3.01 <sup>b</sup> ± 0.04	2.42 <sup>a</sup> ± 0.00	2.64 <sup>a</sup> ± 0.06	<LOQ
		2.54 <sup>a</sup> ± 0.02	2.40 <sup>a</sup> ± 0.00	2.62 <sup>a</sup> ± 0.06	<LOQ
Lactic acid					
0		<LOQ	<LOQ	<LOQ	<LOQ
	1	1.41 <sup>a</sup> ± 0.40	11.34 <sup>b</sup> ± 1.02	11.38 <sup>b</sup> ± 0.19	10.80 <sup>b</sup> ± 0.46
		6.15 <sup>a</sup> ± 0.89	11.74 <sup>b</sup> ± 0.31	11.77 <sup>b</sup> ± 0.98	11.38 <sup>b</sup> ± 0.20
		8.87 <sup>a</sup> ± 0.62	11.85 <sup>b</sup> ± 0.37	12.05 <sup>b</sup> ± 0.84	11.53 <sup>b</sup> ± 0.95
Acetic acid					
0		<LOQ	<LOQ	<LOQ	<LOQ
	1	1.48 <sup>a</sup> ± 0.77	1.07 <sup>a</sup> ± 0.30	1.10 <sup>a</sup> ± 0.45	1.75 <sup>a</sup> ± 0.17
		1.70 <sup>b</sup> ± 0.33	1.06 <sup>a</sup> ± 0.38	1.63 <sup>b</sup> ± 0.40	1.87 <sup>b</sup> ± 0.52
		1.76 <sup>b</sup> ± 0.32	1.20 <sup>ab</sup> ± 0.60	1.66 <sup>b</sup> ± 0.28	1.85 <sup>b</sup> ± 0.36

<sup>a-c</sup>Different superscript letters within same line indicate significant difference (Duncan  $p < 0.05$ ) Results are given mean  $\pm$  SD Abbreviations: SD-C, control sourdough; SD-1, experimental sourdoughs inoculated with *L. plantarum* XL23; SD-2 experimental sourdoughs inoculated with *F. sanfranciscensis* RL976; SD-3, experimental sourdoughs experimental sourdoughs inoculated with inoculated with *L. plantarum* XL23 + *F. sanfranciscensis* RL976.

3.73–3.85 and 14.10–17.00 mL 0.1 N NaOH/10 g dough, respectively. Doughs inoculated with mono- or dual-culture of *L. plantarum* XL23 reached pH values < 4.0 in 12 h. At 24 h, all of the inoculated sourdoughs reached pH values around 3.75 and were stable until the last refreshment. Conversely, in the control sourdough, the pH decreased very slowly and reached similar pH values with the inoculated sourdoughs after 48 h. After 12 h, TTA of the inoculated sourdoughs was determined to be in the range of 8.25–11.75 mL 0.1 N NaOH/10 g dough. The dough inoculated with mono-culture of *L. plantarum* XL23 reached the highest acidity value of 11.75 mL 0.1 N NaOH/10 g dough. Among the inoculated samples, the lowest acidity at 12 h was determined in the SD-2 dough inoculated with *F. sanfranciscensis* RL976 as 8.25 mL 0.1 N

NaOH/10 g dough. After 24 h, the acidity of the inoculated sourdoughs was in the range of 15.35–16.03 mL 0.1 N NaOH/10 g dough. On the other hand, the acidity values of the control dough confirmed the trend shown by pH. TTA increased after 48 h to 13.05 mL 0.1 N NaOH/10 g dough. At the last refreshment, the highest acidity was determined in the SD-3 sourdough produced with the dual-culture inoculum. Fast acidification is very important especially at the industrial scale to conduct sourdough fermentation in a shorter time. In the present study, experimental sourdoughs produced with mono- and dual-culture showed faster acidification compared to control sourdough produced spontaneously. In a previous study, experimental sourdoughs were produced by using mono- and dual-starter culture combinations of *L. plantarum* and *F. sanfranciscensis* and at the end of the fermentation, pH and TTA values were in the range of 3.44–4.09 and 10.10–12.60 mL, respectively (Ventimiglia et al., 2015).

The results of the cell counts on MRS agar are shown in Fig. 2b. After 2 d, LAB counts reached more than 9 log CFU/g in all sourdoughs. All of the presumptive LAB counts were increased and were in the range of 9.17–9.85 log CFU/g at the last refreshment. In the unfermented doughs, yeast and mold counts were 3 and 2.47 log CFU/g, respectively (Fig. 2c). At the 3rd d of refreshment, mold growth was not detected on any of the agar media. Yeast counts exhibited variations. On the first day, yeast growth was not observed on agar media; however, some of the sourdoughs showed different patterns through every refreshment. For back-slopping, boiled and cooled water was added and thus, water cannot be the source of microorganisms. On the other hand, flour addition at every refreshment step can be the source of species other than inoculated LAB strains. However, when the strains adapted to the sourdough environment, they dominate the microbiota. Presumptive yeast counts were 1.30 and 4.46 log CFU/g in the control and SD-1 sourdough samples at the last back-slopping stage, respectively. At the beginning of the fermentation total mesophilic aerobic microorganisms were determined as 3.7 log CFU/g in unfermented doughs. All of the total mesophilic aerobic counts were increased on the first day and were in the range of 9.18–9.70 log CFU/g at the last refreshment as shown in Fig. 2d. Presumptive total coliform bacteria counts of the sourdoughs were 120 MPN/g at the beginning of the fermentation. On the first day of refreshment, the presumptive coliform group bacteria was < 3 MPN/g in the inoculated sourdoughs. In the control sourdough, presumptive total coliform bacteria counts were decreased after 2 d. As it can be seen, control sourdough reached the characteristics of the inoculated sourdough after 48 h. The disappearance of the presumptive coliform bacteria can be related to the pH decrease. In the inoculated sourdoughs, acidification was faster than control dough.

Sugar, organic acid and ethanol contents of produced experimental sourdoughs were investigated and the results are given in Table 2. It was reported that strain-specific interactions with other species and strain-dependent differences in the utilization of carbohydrate metabolism could be observed during sourdough fermentation (Fraberger et al., 2020; Rogalski et al., 2021a). In the present study, differences in sugar consumption and lactic acid production during sourdough fermentation were observed in the inoculated sourdoughs compared to the control dough. At the end of the last refreshment, maltose + sucrose, glucose and fructose contents of the inoculated sourdoughs were lower than those registered in the control dough. Consequently, lactic acid production was higher in the inoculated sourdoughs. In the present study, the selected strains were isolated from sourdoughs, thus, they are already adapted to this fermentation environment. Inoculated sourdoughs were characterized by high LAB counts, fast acidification and low pH values. At the first refreshment, pH values of the inoculated doughs were decreased below 4.0. On the other hand, the sourdough sample produced without starter culture addition reached similar pH levels at the 2nd refreshment. Acidity values and LAB counts of the samples confirmed the trend shown by pH. After two days the control sourdough exhibited the same patterns as the inoculated sourdoughs.

During sourdough fermentation, various VOCs are generated

**Table 3**  
VOCs in the experimental sourdough samples as relative peak area (%).

Chemical compounds <sup>a</sup>	Samples				
	D-0 <sup>b</sup>	SD-C <sup>c</sup>	SD-1 <sup>c</sup>	SD-2 <sup>c</sup>	SD-3 <sup>c</sup>
2-Octen-1-ol (E)	10.64	n.d.	n.d.	n.d.	n.d.
2-Penten-1-ol	n.d.	n.d.	n.d.	0.42	n.d.
2-pentyl-furan	n.d.	0.61	1.42	0.86	1.33
3-methyl-butanol	7.51	n.d.	2.41	n.d.	n.d.
4-amino-1-Pentanol	2.26	n.d.	n.d.	n.d.	n.d.
Acetaldehyde	n.d.	n.d.	1.42	7.39	7.51
Acetic acid	n.d.	7.02	n.d.	n.d.	23.41
Butyl acetate	n.d.	1.29	n.d.	n.d.	n.d.
Ethenyl acetate	n.d.	n.d.	n.d.	21.58	19.88
Benzene, 1,3-dichloro-	1.99	n.d.	n.d.	0.39	0.40
Cyclobutanol	2.80	n.d.	n.d.	0.59	0.39
Cyclopentanol	3.65	n.d.	n.d.	2.18	1.61
D-Limonene	n.d.	14.34	12.18	26.84	23.47
Ethyl Acetate	n.d.	63.06	65.98	n.d.	n.d.
Ethenyl formate	5.17	n.d.	n.d.	1.34	0.87
γ-Terpinene	n.d.	n.d.	0.56	0.58	0.55
Heptanal	n.d.	7.55	3.39	12.58	n.d.
Hexanal	56.55	5.70	1.83	21.56	0.08
Humulene	n.d.	n.d.	0.35	0.72	3.26
o-Cymene	n.d.	0.43	0.73	1.62	1.69
Pentanal	9.43	n.d.	n.d.	n.d.	n.d.
Pentane	n.d.	n.d.	9.73	n.d.	12.48
trans-1,2-Cyclopentanediol	n.d.	n.d.	n.d.	1.35	3.07

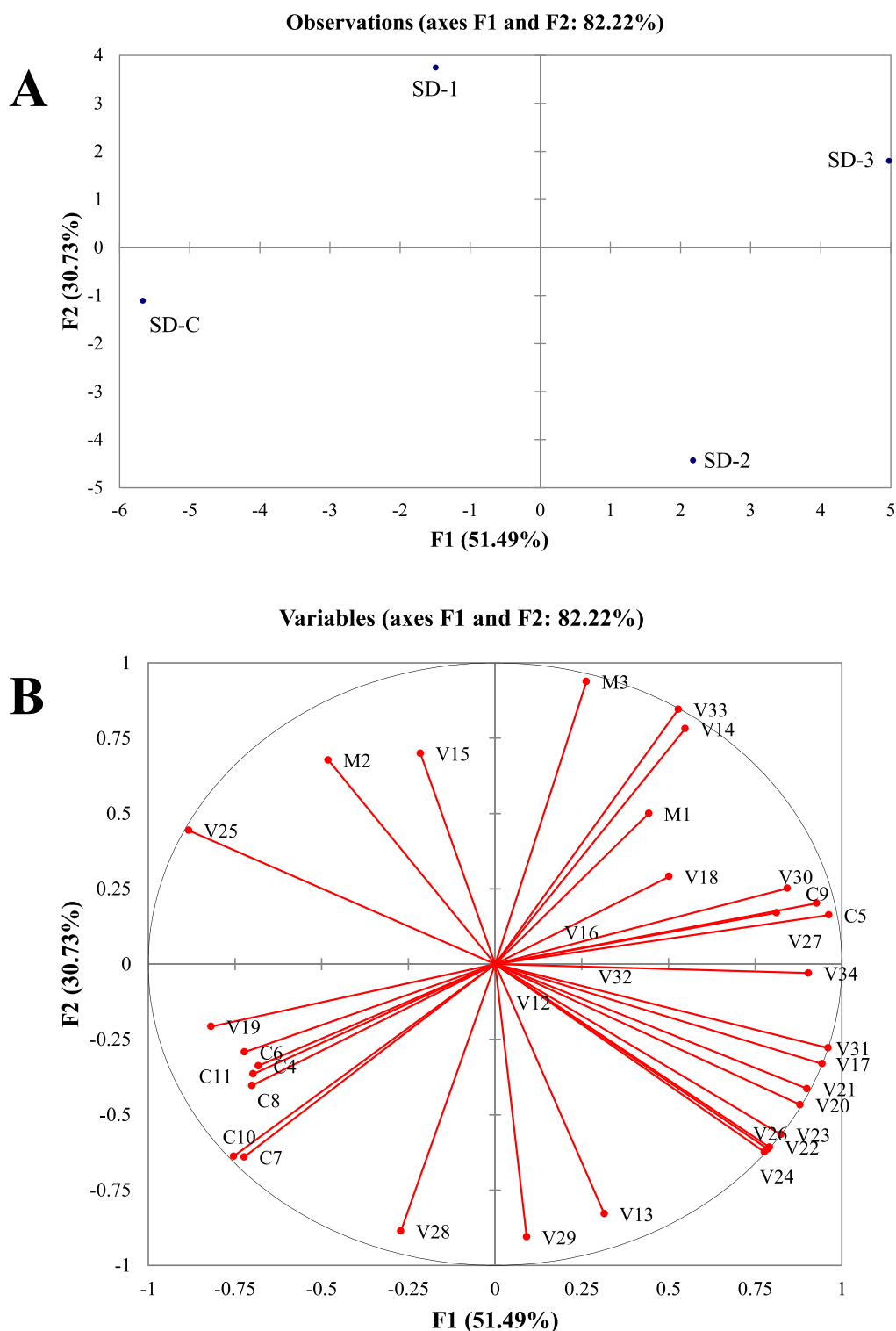
Abbreviations: n.d., not detected; D-0, dough soon after production; SD-C, control sourdough; SD-1, experimental sourdoughs inoculated with *L. plantarum* XL23; SD-2 experimental sourdoughs inoculated with *F. sanfranciscensis* RL976; SD-3, experimental sourdoughs experimental sourdoughs inoculated with inoculated with *L. plantarum* XL23 + *F. sanfranciscensis* RL976.

<sup>a</sup>Results indicate mean values of two measurements and are expressed as relative peak areas (peak area of each compound/total area) × 100.

<sup>b</sup>Sourdoughs at the final refreshment (3rd day).

depending on the complex and dominant microbiota of the sourdoughs. As reported previously, technological properties and VOCs generation in sourdough fermentations are strongly affected by the added starter culture and fermentation temperature (Siepmann et al., 2019). In addition to microbiota and temperature, the type of wheat flour could influence the generated volatile compounds profile of the final bread (Rehman & Awan, 2012). In the present study, the SPME-GC-MS chromatographic analysis of the experimental sourdoughs revealed the presence of 23 VOC compounds belonging to different chemical groups as shown in Table 3. As reported previously, the synthesis of VOC is clearly species-specific, but, it is evident that LAB including *F. sanfranciscensis* and *L. plantarum* species contributed to higher and wider spectrum of VOC with respect to baker's yeast (Arora et al., 2021). In the present study, D-limonene was detected in all sourdoughs. This compound has been detected also in sourdoughs prepared with chestnut flour, semolina sourdoughs and also, mixed flour blend dough (Alfonzo et al., 2016; Aponte et al., 2013; Arora et al., 2021; Corona et al., 2016; Galoburda et al., 2020). 2-pentyl furan was generated in all experimental sourdoughs and this compound was reported as one of the typical sourdough VOC characterized by a fruity aroma (Montemurro et al., 2020). Some VOC compounds as the metabolite of *L. plantarum* were reviewed (Rehman, Paterson, & Piggott, 2006) and among them, ethyl acetate, acetaldehyde, 3-methyl-butanol and heptanal were also detected in our study. The occurrence of 3-ethyl butanol in wheat flour sourdough fermented with *L. plantarum* was previously reported (Rehman & Awan, 2012). Hexanal is another detected compound in all sourdoughs in the present study. Similarly, Liu et al. (2020) reported the occurrence of hexanal in the *L. plantarum* and *F. sanfranciscensis* inoculated sourdoughs. According to the comprehensive aroma profile study conducted by Liu and others (2020), the two predominant species in sourdough *L. plantarum* and *F. sanfranciscensis* had their distinct flavor profiles.





**Fig. 3.** Score plot (A) and loading plot (B) resulting from principal component analysis of variables determined for sourdoughs. Abbreviations: SD-C, control sourdough; SD-1, experimental sourdoughs inoculated with *L. plantarum* XL23; SD-2 experimental sourdoughs inoculated with *F. sanfranciscensis* RL976; SD-3, experimental sourdoughs experimental sourdoughs inoculated with inoculated with *L. plantarum* XL23 + *F. sanfranciscensis* RL976; M1, MRS; M2, YPD; M3, PCA; C4, pH; C5, TTA; C6, maltose + sucrose; C7, glucose; C8, fructose; C9, lactic acid; C10, acetic acid; C11, ethanol; V12, 2-Octen-1-ol (E); V13, 2-Penten-1-ol; V14, 2-pentyl-furan; V15, 3-methyl-butanal; V16, 4-amino-1-Pentanol; V17, acetaldehyde; V18, acetic acid; V19, butyl acetate; V20, ethenyl acetate; V21, benzene, 1,3-dichloro-; V22, cyclobutanol; V23, cyclopentanol; V24, *D*-Limonene; V25, ethyl Acetate; V26, ethenyl formate; V27,  $\gamma$ -Terpinene; V28, heptanal; V29, hexanal; V30, humulene; V31, *o*-Cymene; V32, pentanal; V33, pentane; V34, *trans*-1,2-cyclopentanediol.

### 3.5. Multivariate statistical analysis of experimental sourdoughs

The microbiological and chemical parameters of experimental sourdough samples were subjected to multivariate analysis to evaluate the differences/variabilities among the samples. Data of the sourdough samples were subjected to principal component analysis with a total of 34 variables including microbiological characteristics, chemical properties and generated VOC compounds. The loading and score plots (Fig. 3) show that an overall 82.22% of the variance was explained by the first component (F1 of 51.49%) and a second component (F2 of 30.73%).

As it can be seen, control sourdough (SD-C) differed from the inoculated sourdoughs along with Factor 1. SD-C sourdough was explained by the higher pH, maltose + sucrose, glucose and acetic acid contents than other sourdoughs. Acidity was the lowest in that sample for the negative correlation of pH and TTA. Control sourdough was also characterized by a lower number of VOCs compared to the inoculated sourdoughs. Among the inoculated sourdoughs, SD-1 differed from SD-2 and SD-3 sourdoughs along with Factor 1. Especially, YPD counts were the highest in that sample and relation was observed in the bi-plot. SD-1 sourdough was characterized with 3-methyl-butanol. On the other hand, SD-2 and SD-3 sourdoughs, inoculated with mono- and dual-culture of *F. sanfranciscensis* RL976 were characterized by a high number of VOCs and acidity level that are related to the heterofermentative metabolism of this species. SD-3 sourdough produced with dual culture was also characterized with the high MRS counts and TTA. It was previously reported that the association of *F. sanfranciscensis*, *L. plantarum* and *Saccharomyces cerevisiae* can be used to produce an equilibrated aroma in wheat sourdough breads (Hansen & Hansen, 1994).

### 4. Conclusion

The present study evaluated the variations during sourdough fermentations in terms of chemical characteristics, microbiological properties and VOC profiles in sourdoughs produced with mono- and dual-culture of *L. plantarum* XL23 and *F. sanfranciscensis* RL976 strains isolated during sourdough fermentations. Compared to control sourdough produced spontaneously, starter culture inoculated sourdoughs were characterized by faster acidification and a higher number of VOCs. Especially, the experimental sourdoughs inoculated with mono- and dual-culture of *F. sanfranciscensis* RL976 were characterized by high MRS counts, high TTA values, high concentration of lactic acid and a high number of VOCs. Rapid acidification is very important to conduct sourdough fermentation in a short time at the industrial scale. According to our results, starter culture inoculation resulted in faster acidification compared to uninoculated control sourdough ensuring reproducibility and stability of industrial productions. In that context, the results provided evidence to accept the hypothesis that starter culture addition ensures faster acidification during sourdough fermentation. In addition, results from this study support also the hypothesis that strains isolated from sourdough environment are the most promising candidates to develop starter cultures. As a matter of fact, these strains are already adapted to the specific sourdough environment and can easily control sourdough fermentations. In conclusion, *L. plantarum* and *F. sanfranciscensis* could be applied as dual starter culture in industrial sourdough production to reach the desired level of acidification and aroma in a short time. The development of starter culture combinations was confirmed to be a winning strategy for industrial applications.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Authors' Contributions

CPBG conceived and designed all of the analysis, collected all of the samples, performed all of the laboratory analysis, conducted all of the data tools and analyses, wrote the manuscript and reviewed the final paper; HE conceived and designed all of the analysis, supervised the sample collection and analysis, contributed all of the data tools, contributed to writing the manuscript and reviewed the final paper; LS contributed data tools, performed Illumina data comments and discussions, contributed to writing the manuscript and reviewed the final paper; BA performed laboratory analysis and reviewed the final paper; RG extracted and evaluated DNA quality for Illumina data analysis and reviewed the final paper; EF performed Illumina data analysis and wrote methods for Illumina analysis; NF reviewed the final paper.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2022.100357>.

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