AEM Accepts, published online ahead of print on 14 March 2014
Appl. Environ. Microbiol. doi:10.1128/AEM.00309-14

Diversity of the lactic acid bacteria and yeast microbiota switching from firm to liquid sourdough fermentation

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ABSTRACT

Four traditional type-I sourdoughs were comparatively propagated (28 days) under firm (dough yield of 160) and liquid (dough yield of 280) conditions to mimic the alternative technology options frequently used for making baked goods. After 28 days of propagation, liquid sourdoughs had the lowest pH and TTA, the lowest concentrations of lactic and acetic acids and free amino acids, and the most stable density of presumptive lactic acid bacteria. The cell density of yeasts was the highest in liquid sourdoughs. Liquid sourdoughs showed a simplified microbial diversity and harbored a low number of strains, which were persistent. *Lactobacillus plantarum* dominated firm sourdoughs throughout time. *Leuconostoc lactis* and *Lactobacillus brevis* dominated only some firm sourdoughs and *Lactobacillus sanfranciscensis* persisted, for some time, only in some firm sourdoughs. *Leuconostoc citreum* persisted in all firm and liquid sourdoughs, and it was the only species detected in liquid sourdoughs at all the times, which was flanked by *Leuconostoc mesenteroides* in some sourdoughs. *Saccharomyces cerevisiae*, *Candida humilis*; *Saccharomyces servazzii, Saccharomyces bayanus/Kazachstania* sp. and *Torulaspora delbrueckii* were variously identified in firm and liquid sourdoughs. One hundred ninety-seven volatile components were identified through PT-SPME/GC-MS. Aldehydes, several alcohols and some esters were at the highest levels in liquid sourdoughs. Firm sourdoughs mainly contained ethyl-acetate, acetic acid, some sulfur compounds and terpenes. The use of the liquid fermentation would change the main microbial and biochemical features of the traditional baked goods, which are manufactured under firm conditions since long time ago.

INTRODUCTION

Sourdough is traditionally used as the leavening agent for bread making. About 30 – 50% of the breads manufactured in European countries require the use of sourdough. In Italy, ca. 200 different types of traditional/typical sourdough breads are manufactured, especially by small or medium sized specialized bakeries (1, 2). During the last two decades, a very abundant literature dealt with sourdough: 818 published items are retrieved from Scopus database (http://www.scopus.com/, November 2013). Nowadays, the use of sourdough is extended also for making crackers, pizza, various sweet baked goods and gluten-free products (3, 4). Most of the studies have undoubtedly demonstrated that sourdough positively influences the sensory, nutritional, texture and shelf-life features of baked goods (3, 5). A microbial consortium, mainly consisting of obligately and/or facultatively hetero-fermentative lactobacilli and yeasts, dominates the mature sourdough (6). The microbial ecology dynamics during rye and wheat sourdough preparation was recently described through high-throughput-sequencing approach targeting DNA and RNA (7). The Operational Taxonomic Units network analysis provided an immediate interpretation of the dynamics. As soon the fermentation started by adding water to the flour, the microbial complexity rapidly simplified, and rye and wheat sourdoughs become dominated by a core microbiota, consisting mainly of lactic acid bacteria (7).

The diversity and stability of the sourdough microbiota depends on a number of ecological determinants, which include technology (e.g., dough yield, % sourdough used as inoculum, salt, pH, redox potential, leavening temperature, the use of baker's yeast, number and length of sourdough refreshments, and chemical and enzyme composition of the flour) (3, 8-12) and not fully controllable (e.g., flour and other ingredients, and house microbiota) parameters (12). Furthermore, the metabolic adaptability to stressing sourdough conditions, the nutritional interactions among microorganisms, and the intrinsic robustness or weakness of microorganisms all influence the stability of the mature sourdough (12). Since these numerous factors, the diverse taxonomy and

74 metabolism that characterize sourdough yeasts and, especially, lactic acid bacteria are not surprising
75 (13, 14).

76 Among technology parameters, dough yield (DY = [flour weight + water weight] x 100/flour
77 weight) markedly influences the progress and outcome of sourdough fermentation, due to the effect
78 on microbial diversity (12, 15). Since flours have different capacities to absorb water, DY mainly
79 deals with dough consistency, and measures the amount of water used in the dough formula. The
80 higher is the amount of water, the higher is the value of DY, having an influence on the acidity of
81 the sourdough (15), and, slightly, on the values of water activity (15, 16). Type-I or traditional
82 sourdough is usually made from firm dough, with values of DY of ca. 150-160. Managing
83 (fermentation, refreshment/back-slopping and storage) of type-I sourdough at industrial scale is
84 somewhat considered time consuming, requires qualified staff, and interferes with microbial
85 stability and optimum performance during bread making. To overcome such limitations, liquid
86 sourdough fermentation was more or less recently introduced as another technology option, also for
87 bakeries that used traditional type-I sourdough (17-20). Therefore, a consistent number of bakeries,
88 especially in Italy, switched from firm to liquid sourdough fermentation aiming, however, at
89 manufacturing the same traditional/typical bread. In view of this technology change, some issues
90 deserve responses. How the diversity and stability of the microbiota is influenced during the
91 switching from firm to liquid sourdough? And consequently, does the liquid sourdough
92 fermentation allow the same biochemical and sensory features compared to firm conditions?
93 Besides, a very few studies (21, 22) considered the effect of DY on the diversity of the sourdough
94 microbiota, and none used the approach of this study and provided an in depth microbial and
95 biochemical characterization.

96 This study considered four firm and mature type-I sourdoughs, which were daily propagated for
97 28 days under firm and liquid conditions to mimic the technology changes that likely occur at an
98 industrial scale. The diversity of the lactic acid bacteria and yeast microbiota was monitored
99 through culture-independent and -dependent methods, and the biochemical features and the profile

of volatile components were determined. Multivariate statistical analyses were used to find correlations between the composition of the sourdough microbiota, the biochemical characteristics, volatile components, and firm or liquid sourdoughs.

MATERIALS AND METHODS

Sourdoughs. Sourdoughs from four artisan bakeries, which are located in the Southern of Italy, were considered in the study. The acronyms used were the following: MA, MB, MC (Matera, Basilicata region) and A (Altamura, Apulia region). At bakery scale, sourdoughs were made and propagated through traditional protocols (sourdough type-I), without use of starter cultures or baker's yeast. Preliminarily, sourdoughs were propagated daily at laboratory level for 7 days under the conditions used by artisan bakeries. This would stabilize the effect of the laboratory environment towards the composition of the sourdough microbiota (23). Table 1 describes the ingredients and technology parameters used for daily back-slopping of sourdoughs, which was lasting 28 days. Liquid propagation was carried out under stirring (150 rpm). Between daily fermentation, sourdoughs were left at 10°C for 16 – 19 h. This corresponds to the most diffused practice at artisanal level, which avoids the disturbance of the refrigeration temperature on the microbial performance (e.g., leavening activity), and allows a slight microbial growth. During time, three batches of each sourdough were collected (every 7 days) at the end of fermentation. I, II, III, IV and V identified sourdoughs after 1, 7, 14, 21 and 28 days of back-slopping. Sourdoughs were cooled down to 4°C and analyzed within 2 h after collection. All the analyses were carried out in duplicate for each batch of sourdough (total of six analyses for each type of sourdough).

Determinations of pH, total titratable acidity, organic acids, and free amino acids. The values of pH were determined by a pH-meter. Total titratable acidity (TTA) was measured on 10 g of dough samples, which were homogenized with 90 ml of distilled water for 3 min in a Bag Mixer 400P (Interscience, St Nom, France), and expressed as the amount (ml) of 0.1N NaOH to achieve the pH 8.3. Lactic and acetic acids were determined in the water-soluble extract of the sourdough. Ten grams of sourdough were homogenized with 90 ml of Tris-HCl 50 mM, pH 8.8, buffer. After

126 incubation (30 min at 25°C, under stirring), the suspension was centrifuged (12,857 × *g*, 10 min,
127 4°C) and the supernatant was analyzed using an ÄKTA Purifier™ system (GE Healthcare Bio-
128 Sciences, Uppsala, Sweden), equipped with a refractive index detector (Perkin Elmer Corp.,
129 Waltham, MA). The fermentation quotient (FQ) was defined as the molar ratio between lactic and
130 acetic acids. The concentration of free amino acids (FAA) of the water-soluble extract was
131 determined using the Biochrom 30 Amino Acid Analyser (Biochrom LTD, Cambridge Science
132 Park, England). A mixture of amino acids at known concentration (Sigma Chemical Co., Milan,
133 Italy) was added with cysteic acid, methionine sulphoxide, methionine sulphone, tryptophan and
134 ornithine, and used as the external standard (24).

135 **Polymerase chain reaction (PCR) amplification and denaturing gradient gel**
136 **electrophoresis (DGGE) analysis.** Ninety milliliters of potassium phosphate 50 mM, pH 7.0,
137 buffer were added to 10 g of sourdough and homogenized for 5 min, and the DNA extraction was
138 carried out as described by Minervini et al. (25). Bacterial DNA was amplified with primers Lac1
139 (5'-AGCAGTAGGGAATCTTCCA-3') and Lac2 (5'-ATTYCACCGCTACACATG-3'), targeting
140 a 340 bp region of the 16S rDNA of *Lactobacillus* group, including *Lactobacillus*, *Leuconostoc*,
141 *Pediococcus* and *Weissella* genera (26). DNA from acetic acid bacteria was amplified with primers
142 WBAC1 (5′-GTCGTCAGCTCGTGTCGTGAGA-3′) and WBAC2 (5′-
143 CCCGGGAACGTATTCACCGCG-3′) targeting the V7–V8 regions of the 16S rDNA, which gave
144 amplicons of approximately 330 bp (27). Normalization of the gels was performed using reference
145 ladders of DNA from pure cultures of *Acetobacter malorum* DSM 14337 and *Gluconobacter*
146 *oxydans* DSM 7145 mixed in equal volumes of the same concentration.

147 DNA from yeasts was amplified with primers NL1 (5'-
148 GCCATATCAATAAGCGGAGGAAAAG-3') and LS2 (5'-ATTCCCAAACAACTCGACTC-3'),
149 corresponding to the D1–D2 region of the 26S rDNA gene (28). The PCR core program was carried
150 out as described elsewhere (26-28).

Amplicons were separated by DGGE using Bio-Rad DCode™ Universal Mutation detection System (Bio-Rad Laboratories, Milan, Italy). Sybr Green I stained gels were photographed through the Gel Doc 2000 documentation system (Bio-Rad Laboratories). Profiles were digitally normalized through comparison with the standard reference (MassRuler™ Low Range DNA Ladder, ready-to-use, 80-1031 bp, Fermentas Molecular Biology tools, part of Thermo Fisher Scientific Inc., Waltham, MA) and BioNumerics software, version 2.50 (Applied Maths, St. Martens-Latem, Belgium). The DGGE bands of yeasts were cut out and eluted in 50 µl of sterile water overnight at 4°C. Two microliters of the eluted DNA were re-amplified and PCR products were separated as described above. Amplicons were eluted from the gel and purified by the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). DNA sequencing reactions were carried out by MWG Biotech AG (Ebersberg, Germany). Sequences were compared using the GenBank database and the BLAST program (29).

Enumeration and isolation of lactic acid and acetic bacteria, and yeasts. Ten grams of sourdough were homogenized with 90 ml of sterile peptone water (1% [wt/vol] of peptone and 0.9% [wt/vol] of NaCl) solution. Lactic acid bacteria were counted using sour dough bacteria (SDB) agar medium, supplemented with cycloheximide (0.1 g liter^{-1}). Plates were incubated under anaerobiosis (AnaeroGen and AnaeroJar, Oxoid, Basingstoke, Hampshire, UK) at 30°C for 48 h. At least ten colonies of presumptive lactic acid bacteria were randomly selected from the plates containing the two highest sample dilutions. Gram-positive, catalase-negative, non-motile rods and cocci isolates were cultivated in SDB broth at 30°C for 24 h and re-streaked onto the same agar medium. All isolates considered for further analyses were able to acidify the culture medium. Acetic acid bacteria were counted on Deoxycholate Sorbitol Mannitol medium (DSM) (30) supplemented with cycloheximide (0.1 g liter^{-1}). Plates were incubated at 37°C for 2-4 days under aerobic conditions. At least five colonies of presumptive acetic acid bacteria were randomly selected from the plates containing the two highest sample dilutions. Gram-negative, aerobic, rod-shaped bacteria were cultivated in DSM broth at 37°C for 2-4 days and re-streaked onto the same

agar medium. Stock cultures were stored at –20°C in 10% (vol/vol) glycerol. The number of yeasts was estimated on Sabouraud Dextrose Agar (SDA) (Oxoid) medium, supplemented with chloramphenicol (0.1 g liter^{-1}) at 30°C for 48 h. Randomly five selected colonies of yeasts from the highest plate dilutions were sub-cultured in SDA and re-streaked onto the same agar media.

Genotypic characterization by Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) analysis. Genomic DNA of lactic acid bacteria and acetic acid bacteria was extracted according to De Los Reyes-Gavilán et al. (31). Three oligonucleotides, P4 (5'-CCGCAGCGTT-3'), P7 (5'-AGCAGCGTGG-3') (32) and M13 (5'-GAGGGTGGCGGTTCT-3') (33), with arbitrarily chosen sequences, were used for biotyping of lactic acid bacteria and acetic acid bacteria isolates. Reaction mixture and PCR conditions for primers P4 and P7 were those described by Corsetti et al. (32), whereas those reported by Zapparoli et al. (34) were used for primer M13.

Genomic DNA of yeast was extracted using Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. Two oligonucleotides M13m (5'-GAGGGTGGCGGTTC-3') and Rp 11 (5'-GAAACTCGCCAAG-3') (35) were used singly in two series of amplification for biotyping of yeasts isolates. RAPD-PCR profiles were acquired by the Gel Doc 2000 Documentation System and compared using Fingerprinting II Informatix^{TM} Software (Bio-Rad Laboratories). Determining the Dice coefficients of similarity and using the UPGMA algorithm evaluated the similarity of the electrophoretic profiles. Since RAPD profiles of the isolates from one batch of each type of sourdough were confirmed by analyzing isolates from two other batches, strains isolated from a single batch were further analyzed.

Genotypic identification of lactic acid, acetic acid bacteria and yeasts. To identify presumptive lactic acid bacteria strains two primer pairs (Invitrogen Life Technologies, Milan, Italy), LacbF/LacbR and LpCoF/LpCoR, were used for amplifying the 16S rDNA (36). Primers designed for the *recA* gene were also used to distinguish *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* species (37). Primers designed for the *pheS* gene were

used to identify at the species level within the genera *Leuconostoc* and *Weissella* (38). Sequencing analysis for acetic acid bacteria was carried out using primers 5′-CGTGTCGTGAGATGTTGG-3′ (position 1071–1087 on 16S rDNA, *Escherichia coli* numbering) and 5′-CGGGGTGCTTTTCACCTTTCC-3′ (position 488–468 on 23S rDNA, *E. coli* numbering), according to the procedure described by Trčekl and Teuber (39). To identify presumptive yeasts two primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') were used for amplifying the divergent D1/D2 domain of the 26S rDNA (40). Electrophoresis was carried out on agarose gel at 1.5% (wt/vol) (Gellyphor, EuroClone) and amplicons were purified with GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare). Sequencing electrophoregrams data were processed with Geneious (http://www.geneious.com). rDNA sequences alignments were carried out using the multiple sequence alignment method (41) and identification queries were fulfilled by a BLAST search (29) in GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Determinations of volatile components and volatile free fatty acids. Volatile components (VOC) were extracted through Purge and Trap (PT) coupled with Gas Chromatography-Mass Spectrometry (PT-GC/MS), according to Di Cagno et al. (42). Volatile free fatty acids (VFFA) were extracted by Solid Phase Micro-Extraction (SPME) coupled to GC/MS (SPME-GC/MS). Prior to PT and SPME analysis, a suspension of 10% wt/wol sourdough in UHQ-desodorized, was homogenized with Ultra-Turrax. For extraction of VOC, 10 milliliters of this suspension were poured into a glass extractor connected to the PT apparatus (Tekmar LSC 3000, Agilent Technologies, Les Ulis, France). Extraction was carried out at 45°C for 45 min with helium at a flow rate of 40 mL/min on a Tenax trap at 37°C. Trap desorption was at 225°C and injection into the chromatograph was performed directly into the column with a cryo-cooldown injector at -150°C. The chromatograph (6890 Agilent Technologies) was equipped with a DB5-like capillary column (RTX5 Restek France, Lisses), 60 m length, 0.32 µm i.d., and 1 µm thickness. The helium flow rate was 2 mL/min, the oven temperature was 40°C during the first 6 min and, then, it was

229 increased at 3°C/min to 230°C. The mass detector (MSD5973, Agilent Technologies) was used in
230 electronic impact at 70 electron volts in scan mode, from 29 to 206 atomic mass. Identification of
231 volatile compounds was done by comparison of experimental mass spectra with spectra of
232 NIST/EPA/MSDC Mass Spectra Database (Royal Society of Chemistry, Cambridge, UK). Semi-
233 quantification was done by integration of one ion characteristic of each compound, allowing
234 comparing the area of each eluted compound between samples. Units were given in arbitrary area
235 unit of characteristic ions. Analyses were duplicated. For SPME extraction of VFFAs, each sample
236 was analyzed three times with three different dilutions. 200 µl or 400 µl or 1 ml of the 10%
237 suspension of sourdough were poured into a 10 mL flask with 100 µl of 2N sulphuric acid and
238 respectively 900 or 700 or 100 µl of UHQ water . The flask was sealed and placed into a bath at
239 60°C for 15 min. A SPME Carboxen/polydimethylsiloxane 75µm fiber (black plain hub, Supelco,
240 Sigma Chemical Co. L'isle d'Abeau, France) was introduced into the flask and held in the
241 headspace for 30 min at 60°C. Then, it was removed and desorbed for 5 min in a splitless
242 chromatograph injector at 240°C. The chromatograph (6890 Agilent Technologies) was equipped
243 with a Carbowax-like capillary column (Stabilwax DA, Restek France, Lisses), 30 m length, 0.32
244 µm i.d., and 0.5 µm thickness. The helium flow rate was 2 mL/min, the oven temperature was
245 120°C during the first min and, then, it was increased at 1.8°C/min to 240°C. The mass detector
246 (MSD5973, Agilent Technologies) was used as described above. Concentrations of VFFAs were
247 calculated from calibration curves established with external standards of acetic, propionic, butyric,
248 pentanoic, hexanoic, heptanoic, octanoic, 2-methyl-propionic, 3-methyl-butyric and 2-methyl-
249 butyric acids (Sigma) and expressed in ppm.

250 **Statistical analyses.** Data of pH, TTA, organic acids, FAA, FQ, and cell density of
251 presumptive lactic acid bacteria, yeasts and acetic acid bacteria were subjected to one-way
252 ANOVA, and pair-comparison of treatment means was achieved by Tukey's procedure at $P<0.05$,
253 using the statistical software Statistica for Windows (Statistica 7.0 per Windows). Data of pH, TTA,
254 organic acids, FQ, FAA, and cell density of lactic acid bacteria were subjected to permutation

analysis using PermutMatrix (43). Cluster analysis of RAPD profiles was carried out using the Pearson's correlation coefficient and only profiles that differed more than 15% were shown. For each sourdough (after 1 and 28 days of back-slopping), culture-independent (DGGE bands of lactic acid bacteria) and culture-dependent (number of species and strains, cell density of lactic acid bacteria and yeasts, and percentages of obligately and facultatively hetero-fermentative lactic acid bacteria) and biochemical characteristics (pH, TTA, organic acids, FAA, and FQ) data were used as variables for principal component analysis (PCA) analyses. All data were standardized before PCA analysis using the statistical software Statistica for Windows. Volatile components that mainly ($P<0.05$) differentiated sourdoughs (after 1 and 28 days of back-slopping) were also subjected to the PCA analysis.

RESULTS

Technology, biochemical and microbiological characteristics. All sourdoughs used in this study were handled at artisan bakeries for manufacturing leavened baked goods (mainly bread) from at least two years. As it is usual in the Southern of Italy, all sourdoughs were made with *Triticum durum* flour (Table 1). The percentage of sourdough used for back-slopping varied from ca. 6.0% (MA) and 11% (A) to 30% (MB and MC). The preliminary daily sourdough propagation at laboratory level (7 days) did not show modification of the sourdough microbiota compared to profiles, which were found after collecting samples from artisan bakeries (data not shown). Further, sourdoughs were propagated under firm (*F*) (DY of 160) and liquid (*L*) (DY of 280) conditions. The time of fermentation ranged between 3 and 6 h, and the temperature was 25°C. Overall, the time of fermentation for sourdoughs was that traditionally in use by artisan bakeries, and it, in part, reflected the percentage of sourdough used during refreshment.

The pH, total titratable acidity (TTA), organic acids, fermentation quotient (FQ), free amino acids (FAA) and cell density of presumptive lactic acid bacteria were determined throughout time (Table S1). Figure 1 shows the permutation analysis based on the above characteristics after

1 (I) and 28 (V) days of back-slopping. Sourdoughs were distributed into two major clusters (A and B). Apart from the type, cluster A grouped firm sourdoughs after 28 days of propagation. They had values of pH, which ranged from 4.29 – 4.33, the highest TTA (11 – 13 ml 0.1 N NaOH/10 g of dough), almost the highest concentrations of lactic and acetic acids (30 – 56 mmol kg^{-1} and 19 – 45 mmol kg^{-1}, respectively) and FAA (525 – 796 mg kg^{-1}), and the lowest number of presumptive lactic acid bacteria (6.59 – 7.72 log CFU g^{-1}). Cluster B grouped firm (I) and liquid (I and V) sourdoughs. Within B, sub-clusters B1 and B2 included firm sourdoughs after 1 day of propagation, with values of pH ranging from 4.27 to 4.38, which corresponded to TTA of 5.7 – 7.1 ml 0.1 N NaOH/10 g of dough. The concentrations of lactic and acetic acids and FAA were 31 – 53 mmol kg^{-1}, 6 – 20 mmol kg^{-1} and 467 – 643 mg kg^{-1}, respectively. The number of presumptive lactic acid bacteria was almost the highest (7.71 – 8.56 log CFU g^{-1}). Contrarily to firm sourdoughs, which were scattered into two major clusters (A and B), liquid sourdoughs after 1 and 28 days of propagation were grouped into the same cluster B, being separated into sub-clusters B3 and B4, respectively. The concentrations of FAA (280 – 389 mg kg^{-1}) and lactic and acetic acids (22 – 42 and 10 – 14 mmol kg^{-1}, respectively) already differentiated liquid from firm sourdoughs after 1 day of propagation. Comparing liquid sourdoughs after 1 and 28 days of propagation, these latter showed lower values of pH (4.20 – 4.22) and an increased concentration of acetic acid (range 30 – 54%), even though the number of presumptive lactic acid bacteria maintained almost constant (7.51 – 8.56 log CFU g^{-1}). The number of yeasts of MAV*L*, MCV*L* and AV*L* (6.5 ± 0.1, 7.2 ± 0.2 and 7.2 ± 0.1 log CFU g^{-1}, respectively) was ca. 2 log cycles higher ($P<0.05$) than that found in the corresponding firm sourdoughs. A similar value (ca. 6.2 log CFU g^{-1}) was found for firm and liquid MB sourdoughs. Compared to lactic acid bacteria and yeasts, the number of acetic acid bacteria was scarcely relevant. Except for MCV*L*, which contained a significantly ($P<0.05$) higher (3.0 ± 0.5 log CFU g^{-1}) number of acetic acid bacteria than that found in the corresponding firm sourdough (1.0 ± 0.2 log CFU g^{-1}), the other firm and liquid sourdoughs did not show significant ($P<0.05$) differences (1.0 - 3.0 log CFU g^{-1}).

Denaturing gradient gel electrophoresis (DGGE) analyses. No differences were found regarding the number and size of amplicons of the *Lactobacillus* group, either between sourdoughs propagated under firm and liquid conditions or during back-slopping (Figure S1A, B). This finding did not reflect the results, which were found through culture-dependent approach. Primers NL1-GC/LS1, targeting the region of 26S rRNA gene of yeasts, were also used (Figure S2A, B). Sequencing of the main bands revealed the presence of *Triticum* sp. (100% of identity) (DNA band "a"), while band "b" remained unknown. The other DNA corresponded to *Saccharomyces cereviasiae* (99%) (band "c"), *Saccharomyces bayanus*/*Kazachstania* sp. (99%) (band "d"), *Kazachstania* sp./*Kazachstania unispora* (99%) (band "e"), and *Candida humilis*/*Kazachstania barnettii* (100%) (band "f"). Although PCR-DGGE analysis was successful for acetic acid bacteria used as reference strains, no DNA amplicons were found with WBAC1/C2 primers.

Typing and identification of lactic acid bacteria. Gram-positive, catalase-negative, non-motile, cocci and rods able to acidify SDB broth (400 isolates) were subjected to RAPD-PCR analysis (Table 2). The reproducibility of RAPD fingerprints was assessed by comparing the PCR products obtained with primers P7, P4 and M13, and DNA extracted from three separated cultures of the same strain. For this purpose, ten strains were studied, and patterns for the same strain were similar at level of ca. 90% (data not shown) as estimated by UPGMA. As shown by cluster analysis of RAPD profiles using UPGMA, the diversity between isolates of the four sourdoughs ranged from ca. 2.5 to 35% (Figure S3, A-D). Strains showing RAPD profiles with a maximum level of diversity of 15% were grouped into the same cluster (15, 9, 11 and 15 clusters were found for MA, MB, MC and A, respectively). Although some clusters grouped isolates from sourdoughs that were back slopped under the same condition, the major part of them clustered regardless of firm or liquid propagation. Sourdoughs harbored the following species: *Leuconostoc citreum* (26 strains), *Lactobacillus plantarum* (10), *Leuconostoc mesenteroides* (7),

Leuconostoc lactis (4), *Weissella cibaria* (3), *Lactoccocus lactis* (3), *Lactobacillus sanfranciscensis* (3), *Lactobacillus brevis* (3), and *Lactobacillus sakei* (1).

Strains belonging to the same species and variously isolated from sourdoughs (firm and liquid) showed different RAPD-PCR profiles. As expected, the microbiota composition of firm and liquid sourdoughs was almost similar after 1 day of propagation. Later, species succeeded or were only found in firm sourdoughs, and strains differed between firm and liquid conditions (Figure 2A-D). Sourdough MA variously, and in some cases occasionally, harbored *Leuc. mesenteroides*, *Leuc. citreum*, *L. plantarum*, *Leuc. lactis*, *Lc. lactis*, and *W. cibaria* (Figure 2A). Apart from firm or liquid conditions, strains of *Leuc. mesenteroides* (strain 1) and *Leuc. citreum* (s1) persisted throughout propagation. Other strains of *Leuc. citreum* (s4 and s5) occurred from 14 and 21 days onwards, only in liquid sourdough. On the contrary, strains of *L. plantarum* (s1) and *Leuc. lactis* (s1) persisted only in firm sourdough. One strain of *Leuc. citreum* (s2) dominated throughout propagation of sourdoughs MB*F* and *L* (Figure 2B). One strain of *L. plantarum* (s1) was identified during late propagation of only firm sourdough. One strain of *L. sanfranciscensis* (s1) persisted up to 14 days only in MB*F*. Among the five species of lactic acid bacteria that were identified in sourdough MC (Figure 2C), strains of *Leuc. citreum* (s2 and s3) dominated the microbiota of both the firm and liquid sourdoughs. Strains of *L. plantarum* (s1), *Leuc. mesenteroides* (s2) and *L. brevis* (s1) were identified only in firm sourdough, whereas *Leuc. mesenteroides* (s1) was found only in liquid sourdough throughout 28 days. Among the six species of lactic acid bacteria, which were identified in sourdough A (Figure 2D), strains of *Leuc. citreum* (s2 and s4) were always dominant. Two strains of *L. sanfranciscensis* (s1 and s2) only persisted in the firm sourdough up to 21 and 7 days, respectively. One strain of *L. plantarum* (s2) was dominant throughout propagation of A*F*. *Leuc. mesenteroides* (strain s2) was identified and persisted only in sourdough A*L*.

Microbial diversity, and biochemical characteristics. Figure 3 shows the Principal Component Analysis (PCA) based on DGGE profiles, number of species and strains of lactic

358 acid bacteria (Table S2), percentage of obligately and facultatively hetero-fermentative lactic
359 acid bacteria species, cell density of lactic acid bacteria and yeasts, and biochemical
360 characteristics (pH, TTA, concentration of lactic and acetic acids, FQ, and FAA) throughout
361 propagation. PCA analysis showed two significant PCs that explained 38.5% (PC1) and 30.82%
362 (PC2) of the total variance of the data. Apart from the type of sourdough, the distribution on the
363 plane was determined by time of back-slopping, and firm or liquid propagation. After 1 day of
364 propagation, firm and liquid sourdoughs were almost in the same zone of the plane, whereas
365 after 28 days they were scattered into two different zones depending on the way of propagation.
366 In particular, liquid sourdoughs were correlated with high number of DGGE bands, high
367 numbers of lactic acid bacteria and yeasts, low number of species and strains, high and low
368 percentages of obligately and facultatively hetero-fermentative species, respectively. The
369 opposite features, which determined the opposite distribution, were shown by firm sourdoughs
370 after 28 days of propagation. The distribution of sourdoughs also reflected the different
371 biochemical characteristics, which agreed with data from permutation analysis (Figure 1, Table
372 S1).

373 **Typing and identification of yeasts and acetic acid bacteria.** After a preliminary
374 morphological screening, one-hundred-thirty-nine isolates of yeasts (ca. 30 for each sourdough)
375 were subjected to RAPD-PCR (Table S3). Cluster analysis of RAPD-PCR profiles revealed a
376 diversity level among isolates, which ranged from 5 to 35% (data not shown). Isolates showing
377 RAPD-PCR profiles with a maximum level of diversity of 10% were grouped in the same cluster
378 (6, 7, 8 and 7 clusters were found for MA, MB, MC and A, respectively). The major part of
379 isolates was grouped based on firm or liquid propagation. The following species were identified:
380 *Saccharomyces cerevisiae* (sourdough MA*F* and *L*) and *Candida humilis* (sourdough MA*L*);
381 *Saccharomyces servazzii* (sourdough MB*F*) and *S. cerevisiae* (sourdough MB*F* and *L*); *S.*
382 *cerevisiae* and *Torulaspora delbrueckii* (sourdoughs MC*F* and *L*); and *S. cerevisiae, C. humilis*
383 (sourdough A*F* and *L*), and *T. delbrueckii* (sourdough A*F*).

384 Gram-negative, oxidase negative, catalase positive, cocci or rods (ca. 140 isolates of acetic
385 acid bacteria) were subjected to RAPD-PCR analysis (data not shown). Cluster analysis of
386 RAPD-PCR profiles revealed a diversity of 7.5 to 40%. Most of isolates were grouped based on
387 firm or liquid propagation. The following species were identified: *Gluconobacter oxydans,*
388 *Acetobacter malorum* and *Gluconobacter* sp. (sourdoughs MA*F* and *L*); *Gluconobacter frauterii*
389 (sourdoughs MA*F*); *G. oxydans* and *Gluconobacter* sp. (sourdoughs MB*F* and *L*); *G. oxydans*
390 and *A. malorum* (sourdoughs MC*F* and *L*) and *G. frauterii* (sourdoughs MC*F*); *G. oxydans* and
391 *A. malorum* (sourdoughs A*F* and *L*), *Gluconobacter* sp. (sourdoughs A*F*), and *G. frauterii*
392 (sourdoughs A*L*).

393 **Volatile components.** Based on the previous results, which showed only a few differences
394 between firm and liquid sourdoughs after 1 day of propagation, volatile components were
395 analyzed only in sourdoughs after 28 days of propagation and using the firm sourdough at 1 day
396 as the reference. One hundred ninety-seven volatile components were identified through PT-
397 SPME/GC-MS, which belonged to various chemical classes. Table 3 shows the volatile
398 components that mainly ($P<0.05$) differentiated sourdoughs. Nevertheless, only some of them
399 may contribute to the aroma of sourdough backed goods, which varies, depending on the odour
400 activity value (44-46). Data were elaborated through PCA analysis (Figure 4A and B). The two
401 PCs explained ca. 60% of the total variance of the data. Firm and liquid sourdoughs differed,
402 and, as determined by the two PCs (factors), were located in different zones of the plane. Along
403 the factor 1 (40.56%), liquid sourdoughs were oppositely distributed with respect to the firm
404 ones at 1 day of propagation. After 28 days of propagation, firm sourdoughs were located at the
405 same distance from the two above groups. Along the factor 2 (20.06%), sourdoughs MB and MC
406 were separated from MA and A. Overall, aldehydes (e.g., 3-methyl-butanal, octanal, nonanal and
407 decanal) (44, 46) were found almost at the highest levels in liquid sourdoughs. The same was
408 found for several alcohols (e.g., 1-butanol, 2-methyl-1-propanol and 3-methyl-1-butanol) (44-
409 46), especially in sourdough MA. Except for ethyl acetate and methyl acetate, which were

410 identified mainly in firm sourdoughs, esters such as propyl acetate, 2-methyl-propyl acetate, 3-
411 methyl-butyl acetate, 2-methyl-butyl acetate and 2-phenylethyl acetate were also the highest in
412 liquid sourdoughs. Also ketones such as 3-octanone and 3-methyl-butanone mainly characterized
413 liquid sourdoughs. Compared to liquid sourdoughs, the firm ones contained higher levels of
414 sulphur compounds (e.g., dimethyl-trisulfide) (47), terpenes (e.g., beta-pinene, camphene, and p-
415 cymene) and furans, benzene derivatives and hydrocarbons. Among the volatile free fatty acids,
416 acetic and caproic acids were found at the highest levels in firm sourdoughs.
417

418 **DISCUSSION**

419 Four traditional type-I sourdoughs were comparatively propagated under firm (DY of 160)
420 and liquid (DY of 280) conditions. What happens to sourdoughs when switched from firm to
421 liquid fermentation? Could the liquid sourdough fermentation be considered as another
422 technology option for making traditional baked goods, keeping constant the characteristics?
423 Although mature and used since at least two years, firm sourdoughs confirmed the fluctuations
424 of some biochemical and microbial characteristics during daily propagation (7, 23). In spite of
425 this and though the number of isolates was probably not exhaustive to describe all the species
426 and strains diversity, the main traits differentiating firm and liquid sourdoughs emerged from this
427 study, and some responses to the above queries were given.
428 The cell density of presumptive lactic acid bacteria and related biochemical features (e.g.,
429 pH, TTA and concentration of organic acids) were affected by the way of propagation.
430 Permutation analysis based on the above parameters almost clearly separated firm and liquid
431 sourdoughs. After 28 days of propagation, firm sourdoughs had slight higher values of pH (4.29
432 – 4.33) compared to the liquid ones (4.20 – 4.22). These differences did not reflect on TTA,
433 which was the highest on firm sourdoughs. Indeed, these latter had the highest concentration of
434 lactic and, especially, acetic acids. Overall, the concentration of acetic acid increased throughout
435 propagation and firm sourdoughs showed the highest increases. Low values of DY amplify the

436 buffering capacity of the flour, thereby slowing down the rate of acidification even in the
437 presence of higher levels of organic acids (15). The synthesis of acetic acid is negatively affected
438 under liquid conditions (21, 48), even though it was found a major number of obligately hetero-
439 fermentative lactic acid bacteria, which, probably, synthesized more ethanol than acetic acid.
440 Despite the above differences, the molar ratio between lactic and acetic acids, and the resulting
441 FQ, was similar between firm and liquid sourdoughs at the end of propagation. Cell numbers of
442 presumptive lactic acid bacteria moderately fluctuated in firm sourdoughs. On the contrary, the
443 numbers were more stable in liquid sourdoughs, probably due to better environmental diffusion
444 of carbohydrates, FAA and other nutrients (49). The cell density of yeasts of most of the liquid
445 sourdoughs was markedly higher than that found in the firm ones. The higher is the water
446 content of the sourdough and the higher should be the growth of yeasts (16). Sequencing of the
447 main bands from DGGE profiles, revealed the presence of *Saccharomyces cerevisiae* and
448 *Saccharomyces bayanus*/*Kazachstania* sp. in almost all sourdoughs. The DNA band
449 corresponding to *S. cerevisiae* was not more detectable from 14 days onwards only for firm
450 sourdough MA. After 28 days of propagation, two new bands appeared in the liquid sourdough
451 MA, one of which corresponded to *Kazachstania* sp./*K. unispora*. *Candida humilis*,
452 *Kazachstania barnettii*, *Kazachstania exigua*, and *S. cerevisiae* are the dominating yeasts in
453 Italian bakery sourdoughs (15). Overall, *S. cerevisiae* is the species of yeasts most frequently
454 isolated in sourdoughs from the Centre and South of Italy (2, 50, 51). Recently, it was shown that
455 the composition of the yeast microbiota differed between artisan bakery and laboratory
456 sourdoughs (23) and the persistence of *S. cerevisiae* might be due to contamination of the bakery
457 environment with commercial baker's yeast. All the firm sourdoughs, which showed decreased
458 numbers of yeasts, had the highest concentration of FAA. The opposite was found for liquid
459 sourdoughs. The consumption of free amino acids by yeasts was previously described during
460 sourdough fermentation (52). Almost the same species of yeasts were identified, and the same
461 information was obtained through culture-dependent approach. The only exceptions were

462 *Saccharomyces servazzii* (sourdough MB*F*) and *Torulaspora delbrueckii* (sourdoughs MC*F* and
463 *L*, and A*F*).

464 Several species of lactic acid bacteria were variously identified during propagation under
465 firm and liquid conditions. Overall, they corresponded to the dominating or frequently identified
466 facultatively and obligately hetero-fermentative species under the low incubation temperatures
467 and continuous back-slopping, which characterize traditional type-I sourdoughs (2, 3, 15).
468 Identification occurred repeatedly and at short intervals (7 days) of time, which should have
469 allowed a reliable detection of the microbial succession. Some species (e.g., *Weissella cibaria*,
470 *Lactococcus lactis* and *Lactobacillus sakei*) and strains were only temporarily found, while
471 others seemed to be representative of the microbiota. Regardless of the types of sourdough, those
472 propagated under liquid conditions showed a simplified microbial diversity during time (Table 2
473 and Figure 2). Further, liquid sourdoughs harbored a low number of strains, which, however,
474 persisted. *Lactobacillus plantarum* dominated in all firm sourdoughs throughout time, but not in
475 the corresponding liquid sourdoughs. Several strains of *L. plantarum* seemed to share phenotypic
476 traits, which determined the capacity to outcompete the contaminating lactic acid bacterium biota
477 (53). *Leuconostoc lactis* and *Lactobacillus brevis* dominated only firm sourdoughs MA and MC,
478 respectively. *Lactobacillus sanfranciscensis* persisted for some time only in some firm
479 sourdoughs (MB and A). Although *L. sanfranciscensis* is considered as a stable inhabitant of
480 traditional type-I sourdoughs, its competitiveness is markedly intra-specific and depends on a
481 number of technology and environment parameters (54, 55). *Leuconostoc citreum* persisted in all
482 firm and liquid sourdoughs. *Leuc. citreum* was also the only species detected in liquid
483 sourdoughs at all the times, which was flanked by *Leuc. mesenteroides* in liquid sourdoughs MC
484 and A. Overall, *Leuconostoc* species well adapt and grow at low temperature (e.g., 10°C) as that
485 used in this study between each back slopping (56). Flour and the house microbiota are the main
486 factors perturbing the microbial stability of the sourdough during propagation (12, 57). During
487 liquid propagation, a less amount of flour is used compared to firm sourdough. This would

488 reduce the influence of bacteria deriving from flour and, more in general, would lead to a less
489 competitive pressure and environment. Under these conditions, all the liquid sourdoughs shift to
490 a microbiota almost exclusively composed of *Leuconostoc* species.

491 Several studies (23, 58, 59) showed that sub-populations of pediococci, enterococci and
492 acetic acid bacteria are also part of the sourdough microbiota under certain conditions of
493 propagation. Theoretically, liquid propagation was considered to be particularly suitable for
494 acetic acid bacteria. Based on this consideration also this microbial group was investigated in
495 this study. Nevertheless, not consistent differences were found between firm and liquid
496 sourdoughs, and, especially, the number of acetic acid bacteria seemed to be irrelevant compared
497 to lactic acid bacteria and yeasts.

498 Overall, the synthesis of VOC is mainly due to the metabolic activities of yeasts and lactic
499 acid bacteria (45, 46). After 28 days of propagation, firm and liquid sourdoughs were scattered
500 according to Figure 4 (see also Table 3), depending on the levels of several VOC, which,
501 together with non-volatile compounds (46), would have an impact on the sensory features of
502 baked goods. Alcohols (e.g., 1-butanol, 2-methyl-1-propanol and 3-methyl-1-butanol), which
503 mainly derived from the metabolism of free amino acids by lactic acid bacteria and, especially,
504 yeasts were at the highest levels in liquid sourdoughs that harbored the highest numbers of yeasts
505 and the lowest levels of free amino acids (44). Some aldehydes (e.g., octanal, nonanal, decanal
506 and 3-methyl-butanal) and the 3-octanone were also at the highest levels in liquid sourdoughs.
507 Firm sourdoughs mainly contained ethyl-acetate, acetic acid and related methyl- and ethyl-
508 acetates, dimethyl-trisulfide and terpenes (e.g., beta-pinene, camphene, and p-cymene) (44-47).
509 Ethyl-acetate and acetic acid are compounds, which markedly affect the flavor of baked goods
510 (46). Besides, all firm sourdoughs contained a higher concentration of FAA compared to liquid
511 sourdoughs. Notwithstanding the contribution of cereal proteases and the metabolism of free
512 amino acids by yeasts, secondary proteolysis by sourdough lactic acid bacteria is another

metabolic activity, which contributes to the development of typical sourdough baked good flavors (60, 61).

A consistent number of bakeries are considering the liquid sourdough fermentation as an effective technology option to decrease some drawbacks associated with the traditional daily back-slopping of firm sourdoughs. This option is also considered for the manufacture of traditional/typical breads. Although only four sourdoughs were considered in this study, the switch from firm to liquid sourdough seemed to consistently modify the composition of the sourdough microbiota, especially regarding lactic acid bacteria, and the related biochemical features. Not expressing a comparative quality assessment, undoubtedly the use of the liquid fermentation would change the main microbial and biochemical features of the traditional/typical baked goods.

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692

693 **Legend to figures**

694 **Figure 1.** pH, total titratable acidity (TTA, ml of 0.1 N NaOH/10 g of dough), lactic and acetic
695 acids (mM), fermentation quotient (FQ), free amino acids (FAA, mg kg^{-1}), and cell density (log
696 CFU g^{-1}) of presumptive lactic acid bacteria (LAB) of the four sourdoughs (MA, MB, MC, and
697 A) daily propagated under firm (*F*) and liquid (*L*) conditions for 1 (I) and 28 (V) days. The
698 ingredients and technology parameters used for daily sourdough back slopping are reported in
699 Table 1. Euclidean distance and McQuitty's criterion (weighted pair group method with
700 averages) were used for clustering. Colors correspond to normalized mean data levels from low
701 (green) to high (red). The color scale, in terms of units of standard deviation, is also shown.

702 **Figure 2.** Species and bacterial strains (s) of lactic acid bacteria identified through the culture-
703 dependent method in the four sourdoughs (MA, MB, MC, and A) propagated under firm (*F*) and
704 liquid (*L*) conditions for 1 (I), 7 (II), 14 (III), 21 (IV), and 28 (V) days. Black and white squares
705 indicate the presence or absence of strains, respectively. The ingredients and technology
706 parameters used for daily sourdough back slopping are reported in Table 1. Panels: A, MA; B,
707 MB; C, MC; and D, A.

708 **Figure 3.** Score plot of first and second principal components after principal component analysis
709 based on profiles of microbial community (number of bands in DGGE profiles of lactic acid
710 bacteria, number of species and strains of lactic acid bacteria, percentage of obligately and
711 facultatively heterofermentative lactic acid bacteria, and cell densities [Log CFU g^{-1}] of
712 presumptive lactic acid bacteria and yeasts), and values of pH, total titratable acidity (mL of 0.1
713 N NaOH/10 g), free amino acids (mg kg^{-1}), lactic acid and acetic acid (mmol kg^{-1}) concentration
714 and the fermentation quotient of the four sourdoughs (MA, MB, MC, and A) propagated under
715 firm (*F*) and liquid (*L*) conditions for 1 (I) and 28 (V) days. The ingredients and technology
716 parameters used for daily sourdough back slopping are reported in Table 1.
717 DGGE LAB, number of bands in DGGE profiles of lactic acid bacteria; LAB species, number of
718 lactic acid bacteria species; LAB strains, number of lactic acid bacteria strains; Hef Fac,

719 facultatively heterofermentative lactic acid bacteria; Hef Obl, obligately heterofermentative
720 lactic acid bacteria; LAB, cell density of presumptive lactic acid bacteria; Yeasts, cell density of
721 yeasts; TTA, total titratable acidity; FAA, free amino acids; FQ, fermentation quotient.
722 **Figure 4.** Score plot (A) and loading plot (B) of first and second principal components after
723 principal component analysis based on volatile components that mainly ($P<0.05$) differentiated
724 the four sourdoughs (MA, MB, MC, and A) propagated under firm (*F*) and liquid (*L*) conditions
725 for 1 (I) and 28 (V) days. The ingredients and technology parameters used for daily sourdough
726 back slopping are reported in Table 1. C2, acetic acid; C6, caproic acid; Me2C3, 2-methyl-
727 propionic acid; 3Mebutanal, 3-methyl-butanal; bzacetald, benzeneacetaldehyde; 2Mepropanol,
728 2-methyl-1-propanol; 3Mebutanol, 3-methyl-1-butanol; 2Mebutanol, 2-methyl-1-butanol;
729 3Me3buten1ol, 3-methyl-3-buten-1-ol; 3buten2one, 3-buten-2-one; 3Me2butanon3, 3-methyl-2-
730 butanone; MC2, methyl acetate; Mbzte, methyl benzoate; EC2, ethyl acetate; PC2, propyl
731 acetate; MP2C2, 2-methyl-propyl acetate; MB3C2, 3-methyl-butyl acetate; MB2C2, 2-methyl-
732 butyl acetate; MB3C6, 3-methyl-butyl hexanoate; PheEC2, 2-phenyl-ethyl acetate; DMTS,
733 dimethyl-trisulfide; 3Mefuran, 3-methyl-furan; 2Hxfuran, 2-hexyl-furan; Ether, diethyl-ether;
734 3EMbenz, 1-ethyl,3-methyl-benzene; 2EMbenz, 1-ethyl,2-methyl-benzene; TriMbenz1, 1,x,y-
735 trimethyl-benzene; TriMbenz2, 1,w,z-trimethyl-benzene; TriMbenz3, 1,3,5-trimethyl-benzene;
736 apinene, alpha-pinene; bmyrcene, beta-myrcene; dcarene, delta-carene; aterpinene, alpha-
737 terpinene; pcymene, p-cymene; MEMbenz, 1(1-methyl-1-ethenyl)-4-methyl-benzene; bpinene,
738 beta-pinene.
739

740 TABLE 1. Ingredients and technology parameters used for daily sourdough back-slopping.

Sourdoughs[a]		Flour (g)[b, c]	Sourdough (g)[c]	Water (g)[c]	Refreshment (%)	Dough yield[d]	Back slopping (time and temperature)[e]
MA	(*F*)	585.9	62.5	351.6	6.25	160	5; 25
	(*L*)	334.8	62.5	602.7	6.25	280	5; 25
MB	(*F*)	437.5	300	262.5	30	160	4; 25
	(*L*)	250.0	300	450.0	30	280	4; 25
MC	(*F*)	437.5	300	262.5	30	160	3; 25
	(*L*)	250.0	300	450.0	30	280	3; 25
A	(*F*)	556.9	109	334.1	10.9	160	6; 25
	(*L*)	318.2	109	572.8	10.9	280	6; 25

741 [a]Sourdoughs are identified with the names of the bakery. Only one step of propagation (daily back
742 slopping) was traditionally used. [b]*Triticum durum*. [c]The amount of each ingredient refers to 1 kg of
743 dough. [d]Dough yield = (flour weight + water weight) x 100/flour weight. [e]The first number indicates
744 the length of back slopping (h); the second number indicates the temperature (°C) of incubation. *F*,
745 firm sourdough (DY of 160). *L*, liquid sourdough (DY of 280).
746

747 TABLE 2. Species of bacteria identified from the four sourdoughs (MA, MB, MC and A) propagated under firm (*F*) and liquid (*L*) conditions for 1
748 (I), 7 (II), 14 (III), 21 (IV) and 28 (V) days. Identification was carried out by 16S rRNA, *recA*, or *pheS* gene sequencing.

Closest relative and identity (%)[a] / number of strains	**Number of cluster[b] / Conditions and time of back slopping**	**Accession number (number of cluster)**
	Sourdough MA	
Lactobacillus plantarum (100%) / 2	**1, 2 /** *F* I, II, III, IV, V; *L* I	gb|JN851804.1 (1, 2)
Leuconostoc citreum (99-100%) / 5	**3, 5, 6, 9, 10 /** *F* I, II, III, IV, V; *L* I, II, III, IV, V	ref|NR_074694.1 (3, 5), gb|JN851752.1 (6), gb|JN851747.1 (9, 10)
Leuconostoc lactis (100%) / 3	**4, 7, 15 /** *F* II, III, IV, V; *L* III	gb|KC545927.1 (4, 15), gb|KC836716.1 (7)
Lactococcus lactis (100%) / 1	**14 /** *F* III	gb|KC692209.1 (14)
Leuc. mesenteroides (99-100%) / 2	**8, 13 /** *F* I, II, III, IV; *L* I, II, III, IV	gb|KC292492.1 (8), gb|JN863609.1 (13)
Weissella cibaria (99-100%) / 2	**11, 12 /** *F* I; *L* I	gb|JN851745.1 (11, 12)
	Sourdough MB	
L. plantarum (100%) / 2	**1, NC[c] /** *F* III, IV, V; *L* III	gb|JN851804.1 (1), gb|JN851776.1 (NC)
Leuc. citreum (99-100%) / 6	**2, 4, 5, 6, 7, 8 /** *F* I, II, III, IV, V; *L* I, II, III, IV, V	gb|KC836690.1 (2), HM058995.1 (4), gb|JN851747.1 (5, 7, 8), gb|JN851752.1 (6)

749

750 TABLE 2 (continue)

Closest relative and identity (%)[a] / number of strains	Number of cluster[b] / Conditions and time of back slopping	Accession number (number of cluster)
	Sourdough MB	
Lactobacillus sanfranciscensis (100%) / 1	**3** / *F* I, II, III; *L* I	gb|JN851759.1 (3)
Lactobacillus sakei (99%) / 1	**NC** / *F* III	gb|KF193896.1 (NC)
Lactobacillus brevis (99%) / 1	**NC** / *F* III	gb|JN863602.1 (NC)
Leuc. mesenteroides (99%) / 1	**9** / *F* IV; *L* III	gb|KF148692.1 (9)
Lc. lactis (99%) / 1	**NC** / *F* III	gb|CP004884.1 (NC)
	Sourdough MC	
L. plantarum (99-100%) / 3	**1, 10, 11** / *F* I, II, III, IV, V; *L* I, II, III, IV, V	gb|JN851775.1 (1), gb|JN851804.1 (10), gb|JN851803.1 (11)
Leuc. citreum (99-100%) / 5	**2, 3, 5, 6, NC** / *F* I, II, III, IV, V; *L* I, II, III, IV, V	gb|KC836690.1 (2, 5, NC), gb|JN851753.1 (3) ref|NR_074694.1 (6)
L. brevis (100%) / 2	**4, 9** / *F* I, II, III, IV, V; *L* I, II, III	gb|JN863602.1 (4, 9)
Leuc. mesenteroides (100%) / 2	**7, 8** / *F* I, II, III, IV; *L* I, II, III, IV, V	gb|KC542404.1 (7), gb|JN863609.1 (8)
W. cibaria (100%) / 1	**NC** / *L* I	gb|JN851745.1 (NC)

751 TABLE 2 (continue)

Closest relative and identity (%)[a] / number of strains	Number of cluster[b] / Conditions and time of back slopping	Accession number (number of cluster)
	Sourdough A	
L. plantarum (99%) / 3	**1, 2, 9 /** *F* I, II, III, IV, V; *L* I, II	gb|GU138593.1 (1, 2), gb|JN851803.1 (9)
Leuc. citreum (99-100%) / 10	**3, 4, 6, 11, 12, 14, 15, NC (3) /** *F* I, II, III, IV, V; *L* I, II , III, IV, V	gb|KF149766.1 (3, 12, 4, 15, NC) gb|KC836690.1 (6, 11, NC) gb|JN851753.1 (4), gb|KF150181.1 (NC)
L. sanfranciscensis (99-100%) / 2	**5, 7 /** *F* I, II, III, IV; *L* I	gb|JN851754.1 (5, 7)
Leuc. lactis (99%) / 1	**8 /** *L* V	gb|KF193923.1 (8)
Leuc. mesenteroides (100%) / 2	**10, 13 /** *L* I, II, III, IV	gb|JN863609.1 (10, 13)

752 [a]Species showing the highest identity (%) to the strain isolated from sourdough. The percentage of identity was that shown by performing multiple
753 sequence alignments in BLAST.
754 [b]Number (in bold) of RAPD-PCR clusters.
755 [c]NC, not clustered.
756 *W*., *Weissella*; *Leuc*., *Leuconostoc*; *L*., *Lactobacillus*; *Lc*., *Lactococcus*.
757 The ingredients and technology parameters used for daily sourdough back slopping are reported in Table 1.
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760
761

762 TABLE 3. Concentration of volatile free fatty acids (ppm) and volatile components (arbitrary unit of area)* (P≤ 0.05) identified in the four
763 sourdoughs (MA, MB, MC and A) propagated under firm (*F*) and liquid (*L*) conditions for 1 (I) and 28 (V) days.

	MAI	**MAV*L***	**MAV*F***	**MBI**	**MBV*L***	**MBV*F***	**MCI**	**MCV*L***	**MCV*F***	**AI**	**AV*L***	**AV*F***
						Volatile free fatty acids						
Acetic acid	514^{cd}	446^{d}	774^{bc}	863^{b}	824^{b}	1040^{a}	459^{d}	643^{c}	1178^{a}	283^{e}	953^{ab}	847^{b}
2-Methyl-propionic acid	0.27^{bc}	0.40^{b}	0.33^{b}	0.20^{c}	0.40^{b}	0.38^{b}	0.20^{c}	0.36^{b}	0.52^{a}	0.08d	0.51^{a}	0.31^{b}
Caproic acid	2.37^{a}	0.96^{c}	2.82^{a}	2.56^{a}	1.38^{b}	2.69^{a}	2.03^{a}	1.86^{ab}	3.13^{a}	0.71^{c}	0.98^{c}	0.90^{c}
						Volatile components						
Acetaldehyde	1,6E+06^{c}	3,5E+06^{b}	2,4E+06^{bc}	1,8E+06^{c}	4,7E+06^{a}	4,8E+06^{a}	3,5E+06^{b}	6,1E+06^{a}	4,7E+06^{a}	1,7E+06^{c}	3,0E+0^{b}	5,7E+06^{a}
Octanal	3,7E+05^{bc}	5,9E+05^{b}	3,6E+05^{bc}	5,6E+05^{b}	6,9E+05^{b}	4,0E+05^{b}	2,9E+05^{c}	7,1E+05^{b}	5,4E+05^{b}	5,0E+05^{b}	1,2E+06^{a}	4,1E+05^{b}
Nonanal	1,4E+06^{c}	2,0E+05^{b}	2,1E+06^{b}	2,0E+05^{b}	2,7E+05^{b}	2,0E+05^{b}	1,2E+05^{c}	3,4E+06^{ab}	2,2E+05^{b}	2,5E+05^{b}	4,0E+05^{a}	2,3E+05^{b}
Decanal	1,2E+05^{c}	4,3E+05^{ab}	3,5E+05^{b}	4,2E+05^{ab}	5,0E+05^{a}	5,1E+05^{a}	2,1E+05^{c}	4,9E+05^{a}	5,1E+05^{a}	3,8E+05^{b}	4,9E+05^{a}	3,7E+05^{b}
2-Butenal (Z)	3,6E+04^{c}	5,0E+04^{b}	3,6E+04^{c}	4,3E+04^{b}	5,2E+0^{a}	4,5E+04^{b}	4,5E+04^{b}	4,6E+04^{b}	3,8E+04^{b}	3,1E+04^{bc}	4,2E+04^{b}	2,5E+04^{c}
2-Pentenal	4,4E+05^{ab}	2,4E+05^{bc}	3,7E+05^{b}	3,0E+05^{b}	2,6E+05^{bc}	2,8E+05^{b}	5,0E+05^{a}	3,2E+05^{b}	4,3E+05^{ab}	4,1E+05^{ab}	2,4E+05^{bc}	2,0E+05^{c}
3-Methyl-butanal	1,7E+05^{c}	6,0E+05^{b}	1,4E+05^{c}	1,5E+05^{c}	2,1E+06^{a}	1,0E+06^{ab}	4,9E+05^{bc}	2,0E+06^{a}	1,3E+06^{ab}	1,5E+05^{c}	7,0E+05^{b}	3,2E+05^{bc}
Benzeneacetaldehyde	2,9E+05^{c}	1,5E+06^{b}	1,0E+05^{c}	2,6E+05^{c}	4,1E+06^{a}	7,7E+05^{bc}	1,9E+05^{c}	2,9E+06^{ab}	9,3E+05^{bc}	2,0E+05^{c}	6,5E+05^{bc}	1,9E+05^{c}
Ethanol	9,7E+07^{b}	2,1E+08^{a}	3,5E+07^{c}	2,2E+08^{a}	3,7E+08^{a}	3,3E+08^{a}	2,7E+08^{a}	3,8E+08^{a}	3,8E+08^{a}	1,6E+08^{a}	1,8E+08^{a}	3,3E+08^{a}
1-Butanol	7,8E+05^{b}	1,2E+06^{ab}	9,3E+05^{b}	9,6E+05^{b}	1,7E+06^{a}	1,7E+06^{a}	8,3E+05^{bc}	1,7E+06^{a}	1,6E+06^{a}	6,8E+05^{c}	7,9E+05^{b}	6,8E+05^{c}

764

765 TABLE 3 (Continue)

	MAI	MAV*L*	MAV*F*	MBI	MBV*L*	MBV*F*	MCI	MCV*L*	MCV*F*	AI	AV*L*	AV*F*
2-Butanol	5,1E+05^{a}	1,4E+05^{a}	8,6E+04^{b}	8,4E+05^{a}	0,0E+00^{c}	0,0E+00^{c}	7,9E+05^{a}	8,8E+03^{bc}	2,6E+04^{bc}	1,5E+05^{a}	2,0E+02^{c}	2,0E+02^{c}
2-Methyl-1-propanol	4,9E+05^{c}	4,5E+06^{b}	2,4E+05^{c}	2,8E+05^{c}	1,3E+07^{a}	6,0E+06^{b}	1,4E+06^{bc}	9,8E+06^{ab}	4,9E+06^{b}	6,1E+05^{bc}	3,6E+06^{b}	4,5E+06^{b}
3-Methyl-1-butanol	7,1E+06^{c}	8,3E+07^{b}	3,2E+06^{c}	2,3E+06^{c}	2,1E+08^{a}	1,1E+08^{a}	1,8E+07^{ab}	1,6E+08^{a}	8,7E+07^{b}	4,2E+06^{c}	8,5E+07^{b}	4,4E+07^{ab}
2-Methyl-1-butanol	1,4E+06^{bc}	1,7E+07^{ab}	7,7E+05^{c}	6,6E+05^{c}	4,0E+07^{a}	2,3E+07^{ab}	4,6E+06^{b}	3,6E+07^{a}	1,9E+07^{ab}	4,5E+05^{c}	9,8E+06^{b}	4,1E+06^{b}
3-Octanone	1,1E+05^{b}	1,3E+05^{ab}	8,7E+04^{bc}	1,0E+05^{b}	1,1E+05^{b}	8,1E+04^{bc}	6,5E+04^{c}	1,3E+05^{ab}	9,7E+04^{b}	1,0E+05^{b}	1,8E+05^{a}	7,5E+04^{bc}
3-Methyl-2-butanone	1,3E+05^{b}	1,3E+05^{b}	7,4E+04^{bc}	1,5E+05^{ab}	1,8E+05^{a}	1,1E+05^{b}	1,1E+05^{b}	1,4E+05^{b}	9,9E+04^{bc}	1,2E+05^{b}	1,3E+05^{b}	5,6E+04^{c}
Methyl acetate	3,1E+05^{bc}	7,0E+05^{b}	6,3E+05^{b}	3,7E+05^{bc}	4,3E+05^{b}	1,5E+06^{a}	5,7E+05^{b}	5,2E+05^{b}	1,4E+06^{a}	2,8E+05^{c}	7,3E+05^{b}	1,4E+06^{a}
Methyl benzoate	3,9E+04^{b}	6,6E+03^{c}	1,0E+04^{bc}	8,8E+04^{a}	1,5E+04^{bc}	1,7E+04^{bc}	3,1E+04^{b}	1,6E+04^{bc}	2,1E+04^{bc}	4,2E+04^{b}	5,5E+03^{c}	9,1E+03^{c}
Ethyl acetate	9,6E+07^{d}	1,8E+08^{c}	1,1E+08^{b}	1,6E+08^{c}	2,9E+08^{a}	3,1E+08^{a}	1,1E+08^{d}	2,8E+08^{b}	2,3E+08^{ab}	1,2E+08^{d}	1,7E+08^{c}	2,1E+08^{b}
Propyl acetate	1,2E+05^{c}	9,0E+05^{b}	1,4E+05^{c}	3,8E+05^{bc}	3,6E+06^{a}	2,6E+06^{a}	2,9E+05^{bc}	3,0E+06^{a}	1,2E+06^{a}	1,4E+05^{c}	8,8E+05^{b}	1,4E+06^{a}
2-Methyl-propyl acetate	4,5E+04^{c}	1,8E+06^{a}	3,3E+04^{c}	6,3E+04^{c}	5,8E+06^{a}	3,8E+06^{a}	1,8E+05^{b}	4,3E+06^{a}	1,6E+06^{a}	5,3E+04^{c}	1,4E+06^{a}	2,0E+06^{a}

766

767 TABLE 3 (Continue)

	MAI	MAV*L*	MAV*F*	MBI	MBV*L*	MBV*F*	MCI	MCV*L*	MCV*F*	AI	AV*L*	AV*F*
3-Methyl-butyl acetate	4,5E+05 ^{c}	2,5E+07^{a}	2,9E+05^{c}	3,1E+05^{c}	5,3E+07^{a}	4,0E+07^{a}	1,9E+06^{b}	4,2E+07^{a}	2,0E+07^{a}	2,5E+05^{c}	3,0E+07^{a}	1,3E+07^{a}
2-Methyl-butyl acetate	1,1E+05^{bc}	4,3E+06^{b}	8,6E+04^{c}	9,7E+04^{c}	1,3E+07^{a}	9,2E+06^{ab}	4,6E+05^{bc}	9,7E+06^{ab}	4,3E+06^{b}	3,9E+04^{c}	2,6E+06^{b}	1,1E+06^{b}
3-Methyl-butyl hexanoate	3,1E+03^{bc}	2,2E+04^{b}	2,0E+02^{c}	3,5E+03^{bc}	7,9E+04^{a}	3,4E+04^{b}	0,0E+00^{c}	7,6E+04^{a}	2,6E+04^{b}	9,4E+02^{c}	6,5E+03^{bc}	2,9E+03^{bc}
2-Phenyl-ethyl acetate	1,3E+05^{d}	5,4E+05^{bc}	3,9E+03^{g}	9,4E+04^{e}	1,3E+06^{a}	2,0E+05^{c}	3,7E+04^{f}	7,3E+05^{b}	3,7E+05^{c}	7,5E+04^{ef}	1,6E+05^{d}	9,0E+04^{e}
Carbon disulfide	1,0E+04^{cd}	1,6E+04^{c}	2,8E+04^{b}	3,0E+04^{b}	1,1E+04^{cd}	2,7E+04^{b}	2,9E+04^{b}	8,7E+03^{d}	2,8E+04^{b}	3,4E+04^{b}	1,9E+04^{c}	6,5E+04^{a}
Dimethyl-trisulfide	4,4E+03^{c}	3,2E+03^{cd}	4,8E+03^{c}	7,6E+03^{b}	4,8E+03^{c}	9,1E+03^{a}	7,6E+03^{b}	4,8E+03^{c}	8,1E+03^{ab}	4,7E+03^{c}	2,1E+03^{d}	9,9E+03^{a}
3-Methyl-furan	2,0E+05^{ab}	3,0E+04^{d}	1,8E+05^{b}	2,1E+05^{ab}	5,3E+03^{e}	2,6E+04^{d}	1,6E+05^{b}	5,1E+03^{e}	8,8E+04^{c}	2,4E+05^{a}	1,9E+04^{d}	4,9E+04^{cd}
2-Hexyl-furan	2,1E+05^{b}	4,3E+05^{a}	2,5E+05^{b}	2,6E+05^{b}	4,5E+05^{a}	1,9E+05^{b}	2,0E+05^{b}	3,0E+05^{b}	2,1E+05^{b}	1,9E+05^{bc}	4,7E+05^{a}	1,4E+05^{c}
Diethyl-ether	4,8E+03^{c}	4,5E+04^{ab}	2,2E+04^{b}	5,9E+03^{c}	5,6E+04^{a}	6,0E+04^{a}	3,8E+04^{ab}	6,1E+04^{a}	6,7E+04^{a}	1,6E+04^{bc}	5,5E+04^{a}	6,5E+04^{a}
Decane	3,1E+05^{cd}	9,6E+05^{b}	2,3E+05^{d}	4,6E+05^{c}	8,1E+05^{b}	3,0E+05^{d}	2,3E+05^{d}	4,2E+05^{c}	3,4E+05^{cd}	7,7E+05^{b}	1,1E+06^{a}	2,5E+05^{d}
Nonadiene1	1,3E+05^{b}	6,3E+04^{d}	1,0E+05^{bc}	1,6E+05^{b}	4,6E+04^{e}	1,2E+05^{bc}	1,3E+05^{b}	4,6E+04^{e}	8,2E+04^{c}	1,8E+05^{ab}	1,4E+05^{b}	3,0E+05^{a}

768

769 TABLE 3 (Continue)
770

	MAI	MAV*L*	MAV*F*	MBI	MBV*L*	MBV*F*	MCI	MCV*L*	MCV*F*	AI	AV*L*	AV*F*
Nonadiene2	1,5E+05^{b}	6,5E+04^{d}	1,2E+05^{c}	1,7E+05^{bc}	5,0E+04^{d}	1,4E+05^{b}	1,5E+05^{b}	5,3E+04^{d}	1,0E+05^{c}	2,0E+05^{ab}	1,4E+05^{b}	3,1E+05^{a}
Ethyl,3-methyl-benzene	1,5E+05^{b}	8,3E+04^{c}	9,5E+04^{c}	2,2E+05^{a}	8,7E+04^{c}	1,6E+05^{b}	1,4E+05^{b}	1,1E+05^{bc}	1,1E+05^{bc}	1,6E+05^{b}	8,8E+04^{c}	1,1E+05^{bc}
1-Methyl,2-ethyl-benzene	2,1E+05^{ab}	1,1E+05^{b}	1,1E+05^{b}	3,5E+05^{a}	9,9E+04^{c}	1,7E+05^{b}	2,0E+05^{ab}	1,3E+05^{b}	1,4E+05^{b}	2,2E+05^{ab}	1,2E+05^{b}	2,1E+05^{ab}
1,2,3-Trimethyl-benzene	2,1E+05^{b}	1,3E+05^{d}	1,4E+05^{cd}	2,9E+05^{a}	1,4E+05^{cd}	2,3E+05^{b}	2,1E+05^{b}	1,8E+05^{bc}	1,6E+05^{c}	2,2E+05^{b}	1,3E+05^{d}	1,6E+05^{c}
1,3,5-Trimethyl-benzene	7,4E+05^{b}	4,1E+05^{c}	4,5E+05^{c}	1,2E+06^{a}	3,7E+05^{c}	7,2E+05^{b}	7,3E+05^{b}	4,9E+05^{c}	5,5E+05^{bc}	8,1E+05^{b}	4,6E+05^{c}	5,8E+05^{bc}
1,2,4-Trimethyl-benzene	2,9E+05^{b}	1,7E+05^{bc}	1,9E+05^{bc}	4,5E+05^{a}	1,5E+05^{c}	2,6E+05^{b}	2,8E+05^{b}	1,9E+05^{bc}	2,3E+05^{b}	3,0E+05^{b}	2,0E+05^{b}	2,2E+05^{b}
Naphtalene	7,2E+05^{c}	8,1E+05^{bc}	9,4E+05^{bc}	7,4E+05^{c}	1,1E+06^{b}	1,0E+06^{b}	6,0E+05^{c}	1,4E+06^{a}	1,5E+06^{a}	1,1E+06^{b}	1,3E+06^{ab}	1,5E+06^{a}
Alpha-pinene	6,3E+04^{d}	1,0E+05^{b}	7,2E+04^{c}	8,0E+04^{c}	9,6E+04^{bc}	6,6E+04^{d}	7,1E+04^{c}	1,5E+05^{a}	7,6E+04^{c}	7,5E+04^{c}	1,6E+05^{a}	6,6E+04^{d}
Camphene	7,1E+04^{e}	7,6E+04^{e}	1,1E+05^{cd}	8,0E+04^{e}	8,7E+04^{e}	1,3E+05^{cd}	7,4E+04^{e}	1,0E+05^{d}	1,1E+05^{d}	4,0E+05^{a}	1,9E+05^{c}	2,8E+05^{b}
Beta-pinene	5,4E+04^{cd}	8,0E+04^{c}	4,7E+04^{d}	8,6E+04^{c}	1,2E+05^{ab}	1,3E+05^{a}	7,8E+04^{c}	1,0E+05^{b}	1,2E+05^{ab}	5,7E+04^{cd}	8,3E+04^{c}	4,1E+04^{d}
Beta-myrcene	3,2E+05^{a}	1,9E+05^{c}	2,5E+05^{b}	3,3E+05^{a}	2,4E+05^{b}	3,2E+05^{a}	3,2E+05^{a}	2,4E+05^{b}	3,0E+05^{a}	2,2E+05^{b}	1,7E+05^{c}	2,4E+05^{b}
Delta-carene	2,8E+04^{c}	4,4E+04^{b}	6,3E+04^{ab}	3,1E+04^{bc}	4,8E+04^{b}	7,1E+04^{a}	2,6E+04^{c}	7,5E+04^{a}	5,6E+04^{ab}	2,9E+04^{c}	3,4E+04^{bc}	4,2E+04^{b}
p-Cymene	3,1E+05^{c}	2,9E+05^{d}	5,1E+05^{a}	3,1E+05^{c}	3,0E+05^{d}	4,4E+05^{b}	2,6E+05^{d}	3,6E+05^{c}	4,9E+05^{b}	2,9E+05^{d}	4,6E+05^{b}	5,3E+05^{a}

771

772 TABLE 3 (Continue)
773

	MAI	MAV*L*	MAV*F*	MBI	MBV*L*	MBV*F*	MCI	MCV*L*	MCV*F*	AI	AV*L*	AV*F*
1(1-methyl-1-ethenyl)-4-methyl-benzene	6,1E+04^{c}	5,5E+04^{cd}	8,8E+04^{a}	5,8E+04^{cd}	6,5E+04^{c}	7,6E+04^{b}	5,0E+04^{d}	8,5E+04^{ab}	7,9E+04^{b}	4,9E+04^{d}	9,0E+04^{a}	9,3E+04^{a}

774 *Only VOC, which showed variation (P≤ 0.05) between the samples were reported.
775 In bold are reported the compounds that, based on the literature data (44-47), may have an impact on the aroma of sourdough backed goods.
776 $^{a-g}$: Data are the mean of three independent experiments and values in the same raw with different superscript letters differ significantly (P≤0.05).
777 The ingredients and technology parameters used for daily sourdough back slopping are reported in Table 1.
778

Figure 1.

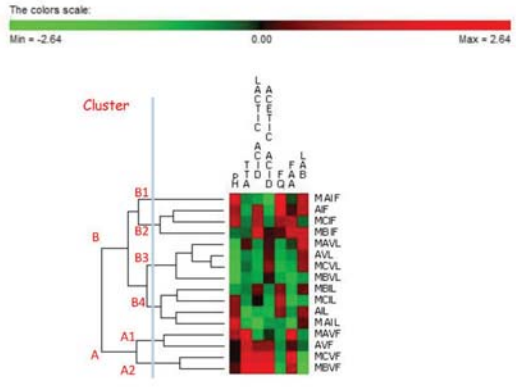
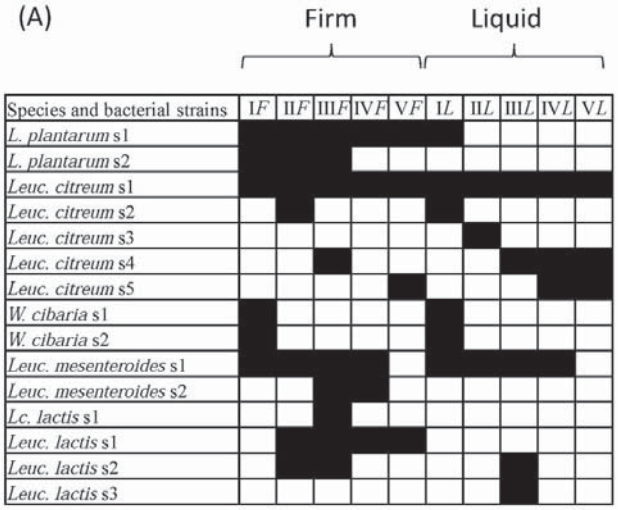
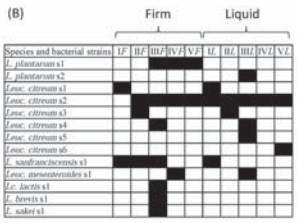


Figure 2.

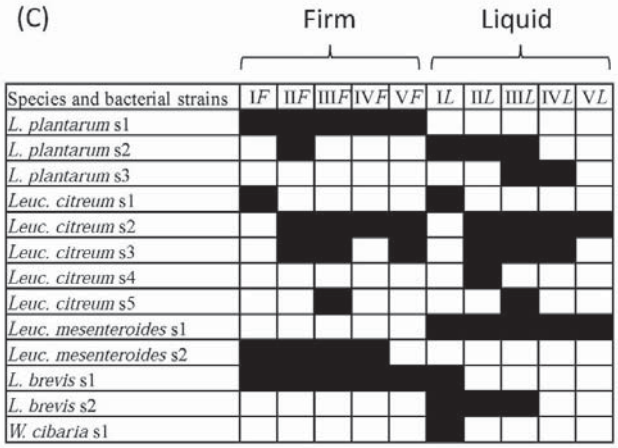
(A)



(B)



(C)



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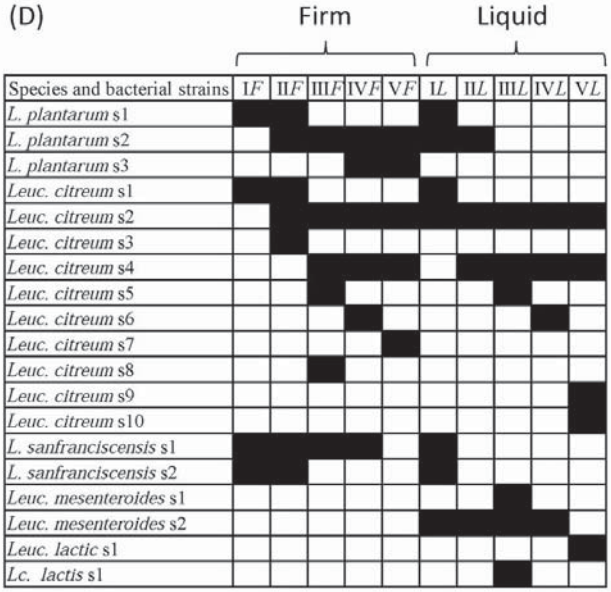

Figure 3.

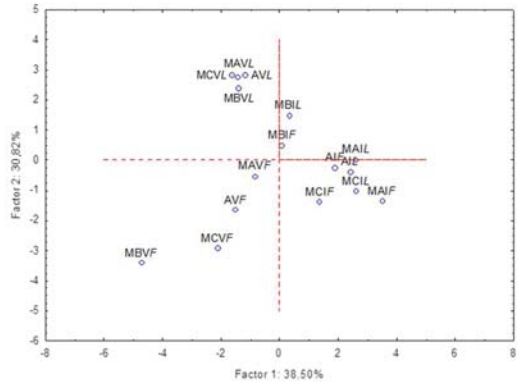

Figure 4.