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Characterization of relative abundance of lactic acid bacteria species in French organic sourdough by cultural, qPCR and MiSeq high-throughput sequencing methods

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ABSTRACT

In order to contribute to the description of sourdough LAB composition, MiSeq sequencing and qPCR methods were performed in association with cultural methods. A panel of 16 French organic bakers and farmer-bakers were selected for this work. The lactic acid bacteria (LAB) diversity of their organic sourdoughs was investigated quantitatively and qualitatively combining (*i*) *Lactobacillus sanfranciscensis*-specific qPCR, (*ii*) global sequencing with MiSeq Illumina technology and (*iii*) molecular isolates identification. In addition, LAB and yeast enumeration, pH, Total Titratable Acidity, organic acids and bread specific volume were analyzed. Microbial and physico-chemical data were statistically treated by Principal Component Analysis (PCA) and Hierarchical Ascendant Classification (HAC). Total yeast counts were 6 $\log_{10}$ to 7.6 $\log_{10}$ CFU/g while LAB counts varied from 7.2 $\log_{10}$ to 9.6 $\log_{10}$ CFU/g. Values obtained by *L. sanfranciscensis*-specific qPCR were estimated between 7.2 and 10.3 $\log_{10}$ CFU/g, except for one sample at 4.4 $\log_{10}$ CFU/g. HAC and PCA clustered the sixteen sourdoughs into three classes described by their variables but without links to bakers' practices. *L. sanfranciscensis* was the dominant species in 13 of the 16 sourdoughs analyzed by Next Generation Sequencing (NGS), by the culture dependent method this species was dominant only in only 10 samples. Based on isolates identification, LAB diversity was higher for 7 sourdoughs with the recovery of *L. curvatus*, *L. brevis*, *L. heilongjiangensis*, *L. xiangfangensis*, *L. koreensis*, *L. pontis*, *Weissella* sp. *and Pediococcus pentosaceus*, as the most representative species. *L. koreensis*, *L. heilongjiangensis* and *L. xiangfangensis* were identified in traditional Asian food and here for the first time as dominant in organic sourdough. This study highlighted that *L. sanfranciscensis* was not the major species in 6/16 sourdough samples and that a relatively high LAB diversity can be observed in French organic sourdough.

1. Introduction

Bread is a staple in many European countries and the production of sourdough breads is part of a cultural and geographical identity (De Vuyst and Neysens, 2005). Despite a number of technical constraints, natural sourdough has advantages such as enhancing bread flavor, prolonging the shelf life, improving the dough structure as well as increasing the nutritional value (Minervini et al., 2014). The microbiota of a stable sourdough principally consists of lactic acid bacteria (LAB) and yeasts (Huys et al., 2013). New species of the genus *Lactobacillus* have been isolated from traditional sourdoughs and various studies on sourdough from different countries have been conducted to isolate a wide range of LAB (De Vuyst and Vancanneyt, 2007). Although they mostly belong to the genus *Lactobacillus*, other genera such as *Leuconostoc*, *Weissella*, *Pediococcus* and *Enterococcus* have also been identified (Gobbetti and Gänzle, 2012). Many species have been found, such as *L. sakei*, *L. crustorum*, *L. nantensis*, *L. mindensis*, *L. reuteri*, *L. brevis*, *L. plantarum*, *L. buchneri*, *L. curvatus*, *L. panis*, *L. pontis*, *L. sanfranciscensis*, *L. spicheri*, *L. kimchi*, *L. amylovorus*, *L. casei*, *W. cibaria*, and *W. confusa* (De Vuyst et al., 2014; Gobbetti and Gänzle, 2012). For yeasts, the six most common yeast species found in stable sourdoughs are *S. cerevisiae*, *Kazachstania exigua*, *Candida humilis*, *Pichia kudriavzevii*, *Torulaspora delbrueckii*, and *Wickerhamomyces anomalus* (De Vuyst et al., 2014). Regarding French ones, *L. plantarum* and *Pediococcus pentosaceus* were found to be dominant (Robert et al., 2009). Other studies revealed a higher diversity with species such as *L. hammesii* and *L. nantensis* (Valcheva et al., 2006, 2005). For French organic and

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conventional sourdoughs, recent studies demonstrated that their microbiota contain mainly *L. sanfranciscensis* (Lhomme et al., 2015a, 2015b) and *Kazachstania bulderi* and *K. unispora* as dominant yeast species (Lhomme et al., 2016). Recently, the rise in culture-independent methods has provided additional data to culture-dependent techniques. Several culture-independent techniques such as pyrosequencing (Bessmeltseva et al., 2014; Ercolini et al., 2013; Lattanzi et al., 2013; Lhomme et al., 2015b), MiSeq ((Minervini et al., 2015), HRM-qPCR (quantitative High Resolution Melting PCR) (Lin and Gänzle, 2014), and quantitative PCR (Lee et al., 2015; Scheirlinck et al., 2009) have been used to study sourdough microbial diversity. The aim of this paper was to analyze quantitatively and qualitatively the LAB relative abundance of organic sourdoughs. A panel of 16 bakers using natural sourdough and organic flours were selected for this study. They were characterized by their practices (farmer-bakers, artisan-bakers and industrial-bakers) and their geographical location. To describe LAB diversity, we combined (i) sourdough isolate identification, (ii) *L. sanfranciscensis* quantitative PCR and (iii) global sequencing with MiSeq Illumina technology.

2. Materials and methods

2.1. Sourdough and bread sampling

Sixteen bakers located in different regions of France were selected (Table 1). They were also chosen because of their bakers' status and related practices: farmer-bakers who produce their own flour (B15, B20, B21, B22, B25 and B27), artisan bakers (B16, B17, B18, B19 and B24) or industrial bakers (B23, B26, B28, B29 and B30). Sourdoughs were collected at the end of the last backslopping as mature sourdoughs (final leavened dough) and final breads were also sampled. Both were stored in sterile vials and conserved at 4 °C until analyses.

2.2. Microbial analysis

For each sample, sourdough was ten-fold diluted in TS (0.1% tryptone, 0.85% NaCl) and mixed for 2 min with a Stomacher (AES Laboratoire, France). Cascade dilutions were performed from 10^{-2} to 10^{-6} and then plated with a spiral plater (Interscience, Saint-Nom-la-Bretèche, France) on MRS5 characterized by a vitamin mix addition (Meroth et al., 2003; Vera et al., 2009) for LAB enumeration and YPD (4 g/L Yeast Extract, 8 g/L glucose, 6.8 g/L agar) for yeast enumeration. Plates were incubated for 48 h at 30 °C under anaerobic conditions (Anaerocult A, Merck, Darmstadt, Germany) for LAB and at 26 °C under aerobic conditions for yeast. After LAB enumeration, approximately 15 bacterial colonies from each sample were selected and, after overnight culture on MRS, maltose (0.05%) and cysteine (1%), these were stored at −80 °C with 40% glycerol. LAB isolates were identified by partial or whole 16S rDNA sequencing. The 16S rDNA (about 1500 bp) of the pure LAB isolates was amplified by PCR as described previously (Jaffrès et al., 2009) from chromosomal DNA using primers fD1 and rD1 (Weisburg et al., 1991). The sequencing primers SP1, SP2, SP3, and SP5 targeting two conserved regions of the 16S rRNA gene were used (Lhomme et al., 2015a). The partial nucleotide sequence (about 700 bp) of the amplified 16S rDNA gene was determined using the sequencing primer SP1. To confirm species group identity, the whole 16S rDNA gene was sequenced (about 1500 bp) using sequencing primers SP. Forward and reverse sequences obtained with SP1 and SP2 or with SP3 and SP5 (Table 2) were concatenated with BioEdit. A similarity of at least 97.6% to 16S rDNA gene sequences of type strains was used as the criterion for identification (Stackebrandt and Ebers, 2006). As the 16S rDNA sequence did not discriminate *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus*, a multiplex PCR targeting the *recA* gene (Torriani et al., 2001) was performed. Three forward primers (para-F, pent-F and F-Plan) specific for each species and a common reverse primer (pREV) were used (Table 2), expecting an amplicon size depending on the species: 107, 218 and 318 bp for *L. paraplantarum*, *L. pentosus* and *L. plantarum*, respectively. In addition, a PCR targeting the *katA* gene (407 bp) was used (Ammor et al., 2005) to discriminate 16S rDNA identification of *Lactobacillus sakei* and *Lactobacillus curvatus* (Table 2). Similarly, as the 16S rDNA sequence did not discriminate all lactic acid bacteria, *rpoA* and *pheS* genes sequencing was performed for *L. koreensis* and *L. heilongjiangensis* identification, using the sequencing primers (Table 2) rpoA-21-F/rpoA-23-R and pheS-21-F and pheS-23-R (Naser et al., 2007). Identification queries were fulfilled by a BLAST search against the National Center for Biotechnology Information (NCBI, Bethesda, USA), the BIBI (Devulder et al., 2003) and the Ribosomal Data Project (Cole et al., 2011) databases.

Single colonies from pure bacterial cultures grown on MRS plates were transferred onto Biolog universal growth (BUG™) agar (Biolog Inc., Hayward,CA) and incubated at 28 °C for 24 h. Colonies were picked using a sterile moistened Biolog cotton swab, suspended in sterile inoculating fluid, IF-A (Biolog Inc., Hayward, CA) and concentration adjusted to match Biolog GEN III turbidity standards. Aliquots of100 μL of bacterial suspensions were loaded into each well of the Microplates. Each bacterial isolate was inoculated on a separate Biolog GEN III MicroPlates™. Readings of inoculated Biolog GEN III microplates was performed at 580 nm (Tecan Infinite Pro 200, Salzburg, Austria) after 24–48 h of incubation at 30 °C. All the wells start out colorless when inoculated. During incubation, there was increased respiration in the wells where cells could use a carbon source and/or grow. Increased respiration causes reduction of the tetrazolium redox dye, forming a purple color. Negative wells remain colorless as did the negative control well with no carbon source. A positive control well was also used as reference for the chemical sensitivity assays.

Table 1
Location and classification of the 16 French bakers selected.

Sourdough	Location of the bakery in France	Bakers' class
B15	Hérault (South)	Farmer baker
B16	Yvelines (Paris area)	Artisan baker
B17	Somme (North)	Artisan baker
B18	Paris	Artisan baker
B19	Aveyron (South)	Artisan baker
B20	Alpes-Maritimes	Farmer baker
B21	Morbihan (West)	Farmer baker
B22	Lot-et-Garonne (South)	Farmer baker
B23	Bas-Rhin (East)	Industrial baker
B24	Vaucluse (South)	Artisan baker
B25	Haut-Rhin (East)	Farmer baker
B26	Côtes-d'Armor (West)	Industrial baker
B27	Puy-de-Dôme (Center)	Farmer baker
B28	Paris	Industrial baker
B29	Aude (South)	Industrial baker
B30	Savoie (East)	Industrial baker

2.3. Determination of TTA (total titratable acidity), pH, bread mass density and organic acids

Sourdough samples were ten-fold diluted with distilled water and mixed with a Stomacher for 2 min (AES Laboratoire, France). Each sample was analyzed in triplicate with an automatic titrator (pH-Matic 23, Grosseron, Saint-Herblain, France), using a 10-mL volume and N/10 NaOH solution concentration. The results of the TTA assay were expressed in volume of 0.1 M sodium hydroxide used to neutralize a 10 g sample (Romanian Standard Methods 90/2007). To measure the bread specific volume, rapeseed density was used. The bread was added in the same container and the excess seed mass was calculated, expressed in dm^3/kg. To measure organic acids, 6 mL of the homogenized mixture was centrifuged at 13,000 rpm for 5 min at room temperature. The samples were clarified by Carrez reagents I and II (250 μL) and centrifuged at 13,000 rpm for 5 min. The supernatant (water-soluble extract) was then analyzed for its organic acid concentration; it was