RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2014) 17:1-9
doi:10.2436/20.1501.01.202 ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

A polyphasic approach to study the dynamics of microbial population of an organic wheat sourdough during its conversion to gluten-free sourdough

Emilie Lhomme,[1] Sandra Mezaize,[2] Maren Bonnand Ducasse,[2] Hubert Chiron,[3] Marie-Christine Champomier-Vergès,[4, 5] Stéphane Chaillou,[4,5] Monique Zagorec,[6,7] Xavier Dousset,[6,7] Bernard Onno[1]*

[1]ONIRIS, Laboratory of Food and Industrial Microbiology, Nantes, France. [2]Biofournil, Le Puiset Doré, France. [3]INRA, UR1268 Biopolymers, Interactions & Assemblages, Nantes, France. [4]INRA, UMR1319 Micalis, Jouy-en-Josas, France. [5]AgroPArisTech, UMR Micalis, Jouy-en-Josas, France. 6INRA, UMR 1014 Secalim, France. [7]LUNAM Université, Nantes, France

Received 18 November 2013 · Accepted 25 February 2014

Summary. To develop a method for organic gluten-free (GF) sourdough bread production, a long-term and original wheat sourdough was refreshed with GF flours. The dynamics of the sourdough microbiota during five months of back-slopping were analyzed by classical enumeration and molecular methods, including PCR-temporal temperature gel electrophoresis (PCR-TTGE), multiplex PCR, and pulsed field gel electrophoresis (PFGE). The results showed that the yeast counts remained constant, although *Saccharomyces cerevisiae*, present in the initial wheat sourdough, was no longer detected in the GF sourdough, while lactic acid bacteria (LAB) counts increased consistently. In the first phase, which was aimed at obtaining a GF sourdough from wheat sourdough, *Lactobacillus sanfranciscensis, L. plantarum*, and *L. spicheri* were the main LAB species detected. During the second phase, aimed at maintaining the GF sourdough, the *L. plantarum* and *L. spicheri* populations decreased whereas *L. sanfranciscensis* persisted and *L. sakei* became the predominant species. Multiplex PCRs also revealed the presence of several *L. sakei* strains in the GF sourdough. In a search for the origin of the LAB species, PCR-TTGE was performed on the flour samples but only *L. sanfranciscensis* was detected, suggesting a flour origin for this typical sourdough species. Thus, while replacement of the wheat flour by GF flour influenced the sourdough microbiota, some of the original sourdough LAB and yeast species remained in the GF sourdough. [**Int Microbiol** 2014; 17(1):1-9]

Keywords: *Lactobacillus* spp. · *Saccharomyces* · *Candida* · sourdough · gluten-free food · organic · lactic acid bacteria · yeast

Introduction

Celiac disease (CD) is a common inflammatory disease of the small intestine that is triggered by storage proteins from wheat, rye, and barley, and it affects about 1 % of the world's population [3]. Currently, the permanent exclusion of gluten from the diet is the only treatment for CD. However, although gluten-free (GF) breads are commercially available, they typically have poor crumble and poor mouth feel and flavor [15]. The use of sourdough, a mixture of flour and water fermented with yeasts and lactic acid bacteria (LAB) [7], may be a "clean label" solution to improve the sensory quality of GF bread. By influencing bread volume, flavor, texture, and staling, sourdough acts on the rheological and organoleptic properties of the dough [2,13]. In addition, the nutritional value and storage

*Corresponding author: B. Onno
ONIRIS, Lab. Microbiologie Alimentaire et Industrielle
Rue de la Géraudière
Nantes Cedex 3, France
Tel. +33-251785535. Fax +33-251785520
E-mail: bernard.onno@oniris-nantes.fr

of sourdough breads are improved by their enhanced mineral bioavailability and delayed starch retrogradation [24]. Moreover, the LAB present in sourdough act to inhibit some of the microorganisms detrimental to the quality and shelf-life of bread. Consequently, sourdough has been proposed as a natural, low-cost, and efficient technology for GF baking [35,40].

The microbiota of sourdough consists of adapted LAB and yeasts [2,19,12], and is frequently dominated by *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* [9] and by the yeasts *Saccharomyces* and *Candida* [20,29]. Although the microbiota of traditional wheat and rye sourdoughs have been well characterized, little is known about the sourdough from alternative GF cereals or pseudo-cereals [51] or about organic sourdough [39]. GF sourdough is generally prepared by spontaneous fermentation [51], commercial starters [34], or selected strains used as starters [50]. In this study, we asked whether the distinctive features of organic wheat sourdough could be maintained by back-slopping with GF flours, while retaining the initial characteristics and specificity of the sourdough. We therefore examined the dynamics of the LAB and yeast from a GF sourdough inoculated with a traditional and stable wheat sourdough, using both PCR combined with temporal temperature gradient gel electrophoresis (PCR-TTGE) and culture-dependent methods, including 16S or 28S rDNA gene sequence analysis. Our aim was to study the population dynamics of the microbiota during its adaptation to a GF sourdough.

Materials and methods

Microbial strains, media, and growth conditions. The type strains *Lactobacillus curvatus* CIP 102992*, L. paraplantarum* CNRZ 188*5, L. pentosus* ATCC 8041, *L. plantarum* ATCC 14917, *L. sanfranciscensis* CIP 103252, *L. spicheri* CIP 108581, *L. sakei* ATCC 15521 and the strains *L. sanfranciscensis* ATCC 43332, and *L. sakei* 23K [4] were used as controls. *L. sanfranciscensis* BF and several strains of *L. sakei* were isolated in this study. LAB were cultured in modified de Man Rogosa Sharpe (MRS) medium [8], MRS4, in which the MRS medium was supplemented with maltose (1 % w/v), fructose (0.5 % w/v), cysteine (0.05 % w/v), and fresh yeast extract (1 % v/v) [25]. The strains were incubated at 30 ºC for 48 h under anoxic conditions (Anaerocult A, Merck, Darmstadt, Germany).

The described strains of *Saccharomyces cerevisiae* and *Candida humilis* were isolated in this study using malt extract agar (Biokar, Beauvais, France) containing chloramphenicol (0.5 g/l) and incubated at 30 ºC for 48 h. For liquid cultures, yeast extract peptone dextrose (YPD) broth was used. The cultures were incubated under aeration overnight at 28 ºC.

For the determination of cell counts in sourdough, 10 g of fresh sourdough was mixed with 90 ml of sterile tryptone salt (TS) solution [0.85 % (w/v) NaCl; 0.1 % tryptone (w/v)] and homogenized for 2 min (Stomacher, AES Laboratories, France). Serial 10-fold dilutions were then plated in triplicate on MRS4 agar and malt extract agar to determine LAB and yeast counts, respectively. After incubation, an average of 20 colonies per sourdough sample were randomly selected. The cultures were further purified and stored at –80 ºC in MRS4 medium glycerol (20 %). Yeasts were stored at –80 ºC in malt extract medium with added glycerol (20 %).

Sourdough fermentation and sampling. An organic wheat sourdough (type I) more than 30 years old was used to prepare an organic GF sourdough by successive back-slopping in a GF environment. Fermentations were carried out with organic GF flours, blending rice (40 %), whole-meal rice (40 %), and buckwheat (20 %) flours with water. The dough yield, defined as the amount of dough obtained from 100 g of flour, was 245. The flours came from two different batches provided by the same supplier. After 12 h of incubation at 25 °C, back-slopping was performed with 25 % of the ripe dough. Theoretically, according to dilution rules, the sourdough had to be refreshed this way six times to become gluten-free. After the dilution step (refreshment steps R0–R8), in which a wheat sourdough was converted to a GF sourdough, maintenance was carried out by back-slopping the sourdough every 12 h during several months under the same conditions. During R0 to R8, samples were drawn from the ripe sourdough every 12 h. From R8 to R328, samples were drawn from the ripe sourdough about every 2 weeks.

Physicochemical analyses. Ten grams of bread or sourdough were mixed with 90 ml of TS solution in a Stomacher for 2 min. Ten ml of the mixture was then homogenized and the pH and total titratable acidity (TTA) were measured with an automatic titrator (pH-Matic 23, Grosseron, Saint-Herblain, France). TTA was expressed as the volume (ml) of 0.1 N NaOH required to adjust the solution to a pH of 8.5. For D- and L-lactic and acetic acid measurements, 10 ml of the homogenized mixture was centrifuged (8,500 rpm, 10 min) at room temperature (Beckman Coulter Genomics, Takeley, Essex, UK) and the supernatant was assayed using the Enzytec kit (Grosseron, Saint Herblain, France), as described in the instruction manual, and by measuring the absorbance at 340 nm (Genesys 10, Grosseron, Saint Herblain, France). Acid amounts were expressed as g/kg of sourdough or bread.

DNA extraction. Bacterial chromosomal DNA was extracted either from the cell pellet of cultures grown in 5 ml of MRS4 or directly from flours and sourdoughs. DNA purification was carried out using a Qiagen DNeasy blood and tissue kit, as described in the instruction manual (Qiagen, SA, Courtaboeuf, France). DNA was extracted from sourdough or flour samples (30 g) as described previously [21].

To extract yeast chromosomal DNA, cells from 3-ml cultures were collected by centrifugation and washed once with 50 mM EDTA, pH 8.0. The collected cells were then broken by vortexing the suspension with 300 mg of glass beads for 3 min at maximum speed in 0.2 ml of lysis buffer (50 mM Tris pH 8.0, 50 mM EDTA, 100 mM NaCl, 1 % SDS, and 2 % Triton X100) with 0.2 ml of phenol. After ethanol precipitation, the DNA was suspended in 50 µl TE and treated with 200 mg RNAse (Roche, Meylan, France)/ml for 30 min at 37 ºC [36]. DNA suspensions were stored at –20 ºC.

PCR-TTGE. For PCR-TTGE, primers V3P2 and V3P3-GC-Clamp were used to amplify the V3 region of 16S rDNA as previously described [21]. Amplicons were analyzed on a 1.5 % agarose gel and visualized by DNA gel staining (SYBR Safe, Invitrogen, Villebon-sur-Yvette, France). The PCR products were subjected to TTGE analysis as described previously [21]. After the run, the gels were stained for 30 min in 300 ml of SYBR Safe 1X, rinsed in Milli-Q water, and visualized by UV illumination.

LAB and yeast identification by 16S and 28S rDNA sequencing. The 16S rDNA (about 1,500 bp) of pure LAB isolates was PCR-amplified using fD1 and rD1 primers [54], as described previously [21]. The amplification was checked by electrophoresis. The partial nucleotide sequen-

ce (about 700 bp) was determined with an automated sequencer (Beckman Coulter Genomics, Takeley, UK) using the internal SP1 primers (not published). Sequences were submitted to the Basic Local Alignment Search Tool program (BLAST) available at the National Center for Biotechnology Information [NCBI, Bethesda, USA http://ncbi.nlm.nih.gov/]. For each species, at least one strain representative of each group was selected and the whole 16S rDNA gene was sequenced (about 1500 bp).

Since the 16S rDNA sequence did not allow discrimination among the various species belonging to the *L. plantarum* group, a multiplex PCR targeting the *recA* gene was performed as described previously [46]. In addition, a PCR targeting the *katA* gene (407 bp) was used, as described previously [1], to confirm the 16S rDNA identification of *L. sakei* species.

The D1/D2 regions (579 bp) of the 28S rDNA gene were PCR-amplified from the chromosomal DNA of yeast isolates using NL1 and NL4 primers [26]. Bacterial identification by sequencing PCR-TTGE DNA fragments: Bands of interest, excised from TTGE gels using a sterile blade, were eluted in 200 µl of Milli-Q water. The eluted DNA (10 µl) was re-amplified with primers V3P2 and V3P3-GC-Clamp. The eluted DNA (10 µl) was then re-amplified with V3P2 and V3P1 primers (V3P3 lacking the GC-Clamp). The PCR products were purified with the MinElute PCR purification kit (Qiagen), ligated into the pCRII-TOPO vector, and cloned in chemically competent *Escherichia coli* TOP10F′ using the TOPO TA cloning kit (Invitrogen, Cergy Pontoise, France). As previously recommended [37], the PCR products were cloned before sequencing in order to avoid the presence of weak bands in addition to the excised bands after re-amplification. For each fragment, about ten clones were selected for DNA extraction and sequencing using the Sp6 and T7 promoters (reverse and forward primers). The PCR-TTGE results were checked to confirm that the profile corresponded with the excised band.

Pulsed field gel electrophoresis. Total DNA from *L. sanfranciscensis* CIP 103252, *L. sanfranciscensis* ATCC 43332, and *L. sanfranciscensis* BF was analyzed by PFGE. The DNA was prepared in agarose plugs as described previously [27]. These DNA plugs were digested in fresh buffer containing 20 units of *SmaI* or *ApaI* (New England Biolabs, Beverly, MA, USA) [43].

Intraspecies genomic diversity of *Lactobacillus sakei*. The PCR cycling conditions have been described previously [4]. The gene content of the strains was described using a two-character matrix (gene markers × isolates) in which 0 indicates a gene marker not detected and 1 the presence of that marker. The data were analyzed using the BioNumerics software, version 6.0 (Applied Maths NV, Sint-Martens-Latem, Belgium). Similarities between the strains were determined by the unweighted pair group method, using the arithmetic averages (UPGMA) clustering method and the character-based Dice similarity coefficient.

Results

Generation of an organic GF sourdough from an organic wheat sourdough: dilution step. Table 1 shows the physicochemical characteristics of the sourdough and of the LAB and yeast microbiota as determined in the initial wheat sourdough and during the dilution step (R0–R8), after successive back-slopping. The pH remained stable during the dilution step whereas the concentrations of L- + D-lactic and acetic acids and the TTA slightly decreased.

The initial population density of yeast in the wheat sourdough was $3.6 \pm 1.2 \times 10^7$ CFU/g (Table 2). Enumeration revealed that two-thirds of the yeast colonies were translucent and rather smooth, while the remaining one-third of the colonies were white, with a mucous appearance. 28S rDNA sequence identification of isolates from these two morphotypes showed that the first type consisted of *C. humilis* and the second type of *S. cerevisiae*. During back-slopping of the dilution step, the total yeast counts increased, from 3.6×10^7 to 6.7×10^7 CFU/g (Table 2), but the proportion of *S. cerevisiae* decreased such that *C. humilis* became the major species.

The initial population density of LAB was $1.3 \pm 0.6 \times 10^8$ CFU/g. Twenty-two isolates were selected from the MRS4 plates and identified by 16S rDNA sequence analysis, resulting in the identification of *L. sanfranciscensis* (n = 10), *L. plantarum* (n = 5), and *L. spicheri* (n = 7) as the predominant LAB species in the initial wheat sourdough. During back-slopping of the dilution step, LAB counts gradually increased, from 1.3×10^8 to 7.4×10^8 CFU/g (Table 2).

The 16S-based identification of the colonies showed that *L. sanfranciscensis, L. plantarum*, and *L. spicheri* persisted but their proportions fluctuated during the dilution step. Therefore, the PCR-TTGE approach was used to compare structural changes in the microbial communities and to monitor the dynamics of the bacterial population. To analyze the TTGE patterns and to detect the presence of bacterial species, fingerprints of the DNA samples obtained from sourdough after each refreshment (12 h) were compared with those of the reference strains. *L. sanfranciscensis* BF, a strain isolated from the sourdough at R76 and identified by 16S rDNA sequencing, was used as a control (Fig. 1).

The results showed that the DNA profiles remained stable during the dilution step (R0–R8) whereas compared with the control strain, *L. spicheri* and *L. plantarum* were not detected by PCR-TTGE even though they had been identified by the culture-dependent method. The profile of each sourdough sample was similar to that of *L. sanfranciscensis* BF but different from the profiles of the *L. sanfranciscensis* reference strains. Thus, by this approach, only *L. sanfranciscensis* was detected in the sourdough. The PFGE profiles of *L. sanfranciscensis* BF were compared with those of *L. sanfranciscensis* ATCC 43332 and *L. sanfranciscensis* CIP 103252 [38] to assess the genetic diversity of this sourdough isolate. Different genetic patterns were obtained for these three strains (data not shown), indicating that strain *L. sanfranciscensis* BF indeed differed from the reference strains, which may also explain its different PCR-TTGE pattern.

Table 1. Organic acids, pH, and total titratable acidity (TTA) in the ripe sourdough at the end of the fermentation step (T0), at the end of the dilution step, and three times during the maintenance step

	L+D [lactic acid] g / kg	[acetic acid] g / kg	FQ[a]	pH[c]	TTA[b,c]
Initial sourdough	6.9 ± 0.1	1.2 ± 0.1	5.8	4.1 ± 0.0	16.0 ± 1.0
Sourdough R7	4.8 ±0.1	0.8 ± 0.1	4.3	4.1 ± 0.01	12.0 ± 0.8
Sourdough R76	3.6 ± 0.2	0.8 ± 0.1	4.5	4.0 ± 0.1	14.0 ± 1.4
Sourdough R114	4.6 ± 0.2	0.5 ± 0.1	9.9	4.2 ± 0.1	10.2 ± 0.6
Sourdough R198	4.2 ± 0.1	0.4 ± 0.1	9.3	4.3 ± 0.01	9.0 ± 1.7
Sourdough R328	5.6 ± 0.2	0.5 ± 0.1	8.4	4.1 ± 0.1	10.4 ± 1.2

[a]Fermentative quotient: [lactic acid] / [acetic acid].
[b]TTA (ml 0.1 N NaOH / 10 g sourdough).
[c]Three independent measurements were performed on each sample for both analyses. The results are presented as mean ± SD.

Characteristics of the organic GF sourdough and the dynamics of its microbiota during the maintenance step. The second step, i.e., maintenance of the sourdough on a GF formulation, lasted 5 months, with regular refreshments twice a day. Samples were drawn from the ripe sourdough about every 2 weeks. The fermentation characteristics of the sourdough are summarized in Table 1. From R76 to R328, the pH remained stable and similar to that of the initial sourdough but the TTA decreased. The concentration of L- + D- lactic acid increased whereas that of acetic acid decreased slightly, leading to a detectable increase in the fermentation quotient. The addition of GF sourdough to the bread formulation increased the fermentation quotient (data not shown), which positively affects the sensorial properties and shelf-life of sourdough bread [44].

As shown in Table 2, the total yeast population decreased slightly, from a maximum of 8.7×10^7 to 5.9×10^7 CFU/g. At the end, *Candida humilis* was the only species detected. The LAB counts remained stable during the maintenance step, at 1.0×10^9 CFU/g, but were about 1 log higher than the counts in the initial wheat sourdough. 16S rDNA identification of the LAB isolates showed that *L. sanfranciscensis, L. plantarum*, and *L. spicheri* were present until R76. *L. sakei* became established beginning at R184 and persisted until R328. Therefore, PCR-TTGE was used to confirm the presence of *L. sakei* and to verify the presence or absence of the other bacterial

Table 2. Microbial sourdough populations of the lactic acid bacteria (*Lactobacillus sanfranciscensis* [Lsan], and *L. spicheri* [Lspi], *L. plantarum* [Lpla] and *L. sakei* [Lsak]) and yeasts (*Candida humilis* [Chum] and *Saccharomyces cerevisiae* [Scer]) during refreshement

	LAB count[a]	Lsan[b]	Lspi[b]	Lpla[b]	Lsak[b]	Yeast count[a]	Chum[b]	Scer[b]
R0 (wheat SD)	1.3×10^8	45 (n = 10)	32 (n = 7)	23 (n = 5)	ND	3.6×10^7	67	33
R1 (day 0)	1.1×10^8	53 (n = 11)	47 (n = 9)	ND	ND	4.5×10^7	67	33
R4 (d2)	2.4×10^8	33 (n = 7)	50 (n = 10)	17 (n = 4)	ND	5.1×10^7	90	10
R8 (d4)	7.4×10^8	29 (n = 6)	71 (n = 14)	ND	ND	6.7×10^7	96	4
R58 (d29)	1.0×10^9	78 (n = 17)	ND	22 (n = 5)	ND	8.7×10^7	100	ND
R76 (d38)	5.3×10^8	42 (n = 7)	16 (n = 3)	42 (n = 7)	ND	7.6×10^7	100	ND
R184 (d92)	8.1×10^8	ND	ND	ND	100 (n = 19)	6.0×10^7	100	ND
R198 (d99)	1.0×10^9	ND	ND	ND	100 (n = 22)	5.9×10^7	100	ND

ND: Not detected.
[a]Expressed as CFU/g
[b]Expressed as a percentage. The number of bacterial isolates identified are shown in parenthesis.

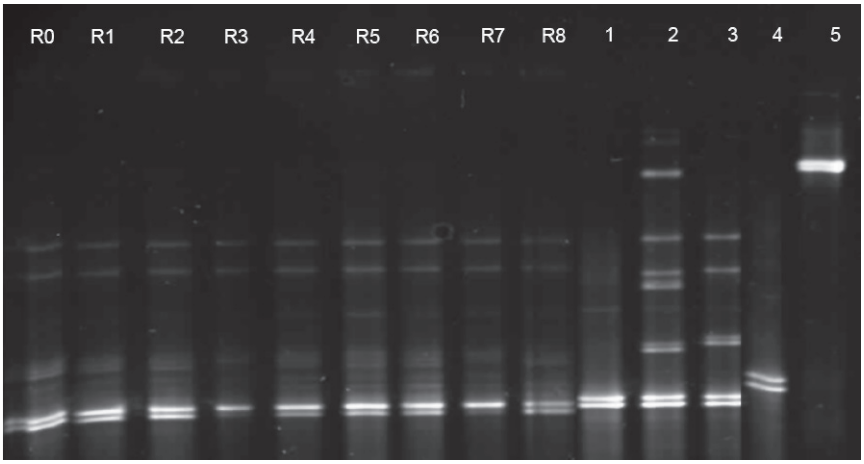

Fig. 1. PCR-TTGE profiles during dilution of the wheat sourdough to the GF formulation. PCR-TTGE profiles were obtained with the primer pair V3P2 and V3P3. R0–R8: profiles obtained with DNA extracted from sourdough at each refreshment step. Control strains: 1: *L. sanfranciscensis*^{T} CIP 103252; 2: *L. sanfranciscensis* ATCC 43332; 3: *L. sanfranciscensis* BF; 4: *L. spicheri*^{T} CIP 108581*;* 5: *L. plantarum*^{T} ATCC 14917.

species. Figure 1 shows the fingerprints of the DNA samples of sourdough after each refreshment compared with those of the reference strains. The DNA of the type strain *L. sakei* ATCC 15521 was added as a control and its profile was compared with that of the *L. sakei* isolates in the GF sourdough. All *L. sakei* PCR-TTGE profiles were similar (data not shown).

The PCR-TTGE profiles in Fig. 2 showed that the microbiota of the sourdough evolved during maintenance. After remaining stable, with identical profiles (R30–R114), a major band appeared beginning at R184 (92 days; band **d**, R184). By comparing band migration positions, band **d** was assigned to *L. sakei*, as subsequently confirmed by cloning and sequencing. The presence of this band correlated with the culture-dependent detection of *L. sakei*. In addition, PCR-TTGE highlighted the stability of *L. sanfranciscensis* in the GF sourdough, as evidenced by a pattern similar to that of *L. sanfranciscensis* BF, which was observed throughout the refreshments, except at R184 and R198. This species was not identified at the latter two steps by culture-dependent methods. Finally, beginning at R266 (133 days) onwards, the sourdough became stable (Fig. 2).

Detection of bacterial species in organic flours by PCR-TTGE. Analysis by PCR-TTGE of the flours used at the beginning and end of back-slopping revealed, in wholemeal rice and rice flours, a single major band and many minor bands, whereas in the buckwheat flours only one major band was detected (Fig. 2). For each flour, there were few differences between the two batches tested. Band **a** was identified as rice chloroplast and band **b** as *L. sanfranciscensis,* although its migration differed from that of *L. sanfranciscensis* BF. Since the other bands were very weak, they were not cloned for sequencing and identification. Thus, while the results indicated the presence of *L. sanfranciscensis* in the rice flour, no such evidence was available for *L. sakei*, as neither the sequences nor the migration patterns of bands from the flour samples were similar to those of known *L. sakei* isolates.

Diversity of *Lactobacillus sakei* in organic GF sourdough. The appearance and maintenance of *L. sakei* observed by the plating method and PCR-TTGE raised the question of the nature of this population and its implantation. As this species has already been described, in both sourdough [33] and in rice [23,41], we wondered whether one or several strains had derived from the bread flours and flourished in the GF sourdough. Taking advantage of our ability to discriminate among *L. sakei* isolates [5,28], the nature of the *L. sakei* population in the sourdough after R184 was examined. Multiplex PCR targeting several gene markers was used to determine the diversity of ten *L. sakei* isolates from R184 and R198. Based on markers described previously [5] a first subset of six gene markers (DrsB, FGP21-0001, FGP332-0009, FGP332-0010, LSA0727, and SspA) was used to assign the genotype clusters of the isolates and a second subset of another six gene markers (LSA0219_b, FGP332-0001, FGP332-0002, FGP332-0007, and FGP332-0012) to assess the strain level. The results showed that all isolates of *L. sakei* belonged to the same clus-

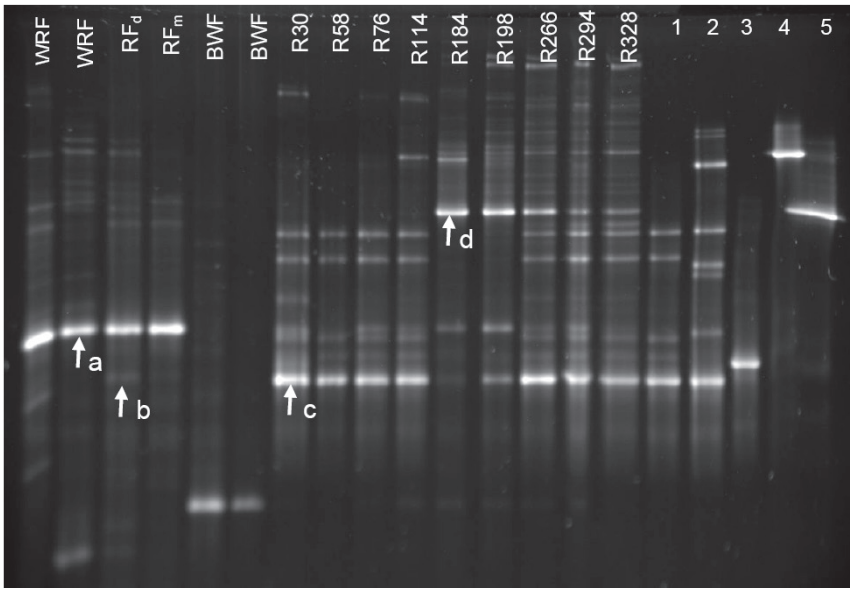

Fig. 2. PCR-TTGE profiles during the maintenance step. Profiles obtained with DNA extracted from flours used for back-slopping are shown: WRF_d, whole-meal rice flours used during the dilution step; WRF_m, whole-meal rice flours used during the maintenance step; RF_d, rice flours used during the dilution step; RF_m, rice flours used during the maintenance step; BWF_d, buckwheat flours used during the dilution step; BWF_m, buckwheat flours used during the maintenance step. R30–R328: Profiles obtained with DNA extracted from GF sourdough at different refreshments (R). Control strains: 1: *L. sanfranciscensis*^{T} BF; 2: *L. sanfranciscensis* ATCC 43332; 3: *L. spicheri*^{T} CIP 108581*;* 4: *L. plantarum*^{T} ATCC 14917; 5 : *L. sakei* ATCC 15521. Bands **a** (choloroplast), **b** and **c** (*L. sanfranciscensis*), and **d** (*L. sakei*) were identified by sequence characterization of the excised fragments.

ter as that of *L. sakei* ATCC 15521, the type strain previously isolated from spoiled rice alcohol [23]. This group is characterized by the presence of the FGP21-0001 marker, which encompasses "non-meat" isolates [5]. Three different profiles were defined, indicating that the *L. sakei* population that arose on GF sourdough after R184 was composed of at least three different strains.

Discussion

This study describes the evolution of the microbiota in an organic GF sourdough derived from an organic wheat sourdough. In the initial long-term wheat sourdough, *C. humilis* was the main species although *S. cerevisiae* was also identified. The results confirm those of a previous study of this wheat sourdough (B. Onno, unpublished), and they are in agreement with previous investigations of sourdough yeasts [9]. In particular, *S. cerevisiae* and *C. humilis* were shown to be associated with LAB in type I sourdoughs [9,17,48]. However, in our study, after back-slopping with rice and buckwheat flours, *C. humilis* was the only yeast species present. Few studies have examined the yeasts in GF sourdough. In a previous study of the development of a commercial starter for the production of GF bread, yeast could not be isolated from the sourdough containing buckwheat [34]. In our sourdough, we recorded a decrease in the *S. cerevisiae* population although this species has been previously described as competitive and as the only species present in rice sourdough [30].

By contrast, *L. sanfranciscensis*, *L. plantarum* and *L. spicheri*, the *Lactobacillus* species most frequently isolated from sourdough [7,11,30,53], were detected in the initial wheat sourdough but were not specific for it. Remarkably, the presence of *L. sanfranciscensis* and *L. spicheri* in this particular wheat sourdough has been reported in earlier studies [14,47], which confirms the long-term stability of these strains.

After back-slopping with GF flours, *Lactobacillus* species could no longer be identified by the culture-dependent methods. However, *L. sanfranciscensis* was still identified by PCR-TTGE. The drop in the acetic acids concentration in the sourdough (Table 2) can be attributed to the implantation of homofermentative *L. sakei*. Although *L. spicheri* has been

described as a competitive LAB in rice sourdoughs [30], in our sourdough its presence gradually decreased until, finally, it was undetectable by culture-dependent and -indepenedent methods. In a previous study [51], *L. spicheri* has not predominated in rice sourdough. *L. sakei*, described as a meat-associated bacterium [4,6], is also less frequently found in traditional sourdough. However, this species has been isolated from wheat sourdough [47]; it has been described as the dominant species of spontaneously fermented amaranth [45] and spontaneously fermented buckwheat [33] sourdoughs. In our study, the type and quality of the substrate as well as the microbial interactions during fermentation might y have influenced the microbial populations of the sourdoughs. It is known that the type of flour, the sourdough-generating process, and other related factors strongly influence the composition of sourdough microbiota [10,31]. In addition, the species of wheat flour (*Triticum durum* or *T. aestivum*) may play a key role in both selecting the LAB population and determining the concentration of nutrients required by the microorganisms dominating the sourdough ecosystem [32]. Our study confirms that the nature of the cereals used might influence the competitiveness and interactions of LAB and yeasts in sourdough.

The short-term adaptation of LAB and yeasts to sourdough prepared from cereals, pseudocereals, and cassava has been examined, but the dynamics of adaptation have not been reported [51]. Nevertheless, the authors observed the establishment of a stable sourdough after 13 and 12 days for rice and buckwheat sourdoughs, respectively. In our sourdough, the profile for refreshment after 15 days (R30) was similar to those of previous refreshments, except for the detection of *L. sakei* at R114 (57 days). It might therefore be the case that modifications in the microbiota were detected only after a long period of time.

To our knowledge, ours is the first study to investigate the adaptability of sourdough refreshed with new flours and the long-term evolution of this sourdough. In earlier studies [30,31,34,35,49,50,53], only short-term (about 10–15 days) monitoring has been carried out, when the stability of the examined sourdoughs seemed to have been established. Other studies have analyzed sourdough evolution over several years but with either no or few changes in the process [39,42]. In the present study, specific LAB strains, such as *L. sanfranciscensis* BF, persisted in the GF sourdough. These LAB might have originated from the wheat sourdough or from the bakery environment, and their persistence might have been the result of consistent fermentation variables (temperature, time), which ultimately led to the selection of LAB strains best adapted to the applied process conditions.

To establish a link between the flours used in sourdough generation and the species found in the sourdough, we searched for bacteria in the flours used. Although *L. sanfranciscensis* is considered a key sourdough LAB [16], its origin has not been clearly established. Contrary to most sourdough lactobacilli, which are also inhabitants of other ecosystems (e.g., human and animal intestines), no other habitats are currently known for *L. sanfranciscensis* [18,52]. However, unlike some species, the presence of *L. sanfranciscensis* in flours has yet to be clearly shown [52]. In our case, *L. sanfranciscensis* was detected in rice flour by PCR-TTGE but not by the culture-dependent method of identification. Thus, this species either was non-cultivable under the chosen conditions or, most likely, was subdominant. Further investigations, using different growth conditions (media, temperature, and atmosphere) and quantitative-PCR will be necessary to confirm the presence of this species in rice flour.

The origin of *L. sakei*, a meat-associated bacterium, in cereals has not been researched, although the bacterium has been isolated from sourdough [33,45,47] and from other vegetable substrates such as kimchi [22]. The analysis of ten *L. sakei* isolates showed their close relationship and that they grouped with *L. sakei* ATCC 15521, which has been isolated from rice wine [23]. At least three different strains were isolated from our GF sourdough. Since all *L. sakei* isolates originating from sourdough sources are genetically closely related [5], the adaptation of these strains to the sourdough ecosystem might be readily studied by searching for specific enzymatic activities, such as maltose utilization.

In our polyphasic approach, only *L. sanfranciscensis* was detected by PCR-TTGE, a non-culture-dependent method. Under our experimental conditions, in which the sourdough being back-slopped was kept in the same facility where a conventional sourdough was maintained, the detection of *L. sanfranciscensis* only by the culture-independent method could indicate that this strain was an environmental contaminant. A possible explanation could be that the specific nutritional requirements of that strain were not provided by the chosen medium [19]. Moreover, the PCR-TTGE seemed to yield a specific profile for strain *L. sanfranciscensis* BF. Since the isolation method and PCR-TTGE are subject to different biases, our study confirms the need to simultaneously use culture-dependent and independent methods to study the microbial diversity of the food fermentation process.

Further investigations, including a metagenomic approach, will likely provide a wealth of information of the microbiota of sourdough, including identification of the relevant microorganisms, their genetic content, and the metabolic and

functional potential of the microbial communities to which they belong [22].

Acknowledgements. We thank the Biofournil bakery for their contribution to this study. We thank Jean-Jacques Joffraud for his help with BioNumerics Software, version 6.0, and Florence Valence-Bertel for the PFGE experiments. This work was supported by the French Ministère de l'Agriculture, de l'Alimentation et de la Pêche.

Competing interests. None declared.

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