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Taxonomic Structure and Stability of the Bacterial Community in Belgian Sourdough Ecosystems as Assessed by Culture and Population Fingerprinting▽†

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A total of 39 traditional sourdoughs were sampled at 11 bakeries located throughout Belgium which were visited twice with a 1-year interval. The taxonomic structure and stability of the bacterial communities occurring in these traditional sourdoughs were assessed using both culture-dependent and culture-independent methods. A total of 1,194 potential lactic acid bacterium (LAB) isolates were tentatively grouped and identified by repetitive element sequence-based PCR, followed by sequence-based identification using 16S rRNA and *pheS* genes from a selection of genotypically unique LAB isolates. In parallel, all samples were analyzed by denaturing gradient gel electrophoresis (DGGE) of V3-16S rRNA gene amplicons. In addition, extensive metabolite target analysis of more than 100 different compounds was performed. Both culturing and DGGE analysis showed that the species *Lactobacillus sanfranciscensis*, *Lactobacillus paralimentarius*, *Lactobacillus plantarum*, and *Lactobacillus pontis* dominated the LAB population of Belgian type I sourdoughs. In addition, DGGE band sequence analysis demonstrated the presence of *Acetobacter* sp. and a member of the *Erwinia*/*Enterobacter*/*Pantoea* group in some samples. Overall, the culture-dependent and culture-independent approaches each exhibited intrinsic limitations in assessing bacterial LAB diversity in Belgian sourdoughs. Irrespective of the LAB biodiversity, a large majority of the sugar and amino acid metabolites were detected in all sourdough samples. Principal component-based analysis of biodiversity and metabolic data revealed only little variation among the two samples of the sourdoughs produced at the same bakery. The rare cases of instability observed could generally be linked with variations in technological parameters or differences in detection capacity between culture-dependent and culture-independent approaches. Within a sampling interval of 1 year, this study reinforces previous observations that the bakery environment rather than the type or batch of flour largely determines the development of a stable LAB population in sourdoughs.

Traditional sourdoughs comprise a complex microbial association of lactic acid bacteria (LAB) and yeasts and are thought to improve sensory, texture, and health-promoting properties of many bakery products (15). During sourdough fermentation, the prevailing LAB produce acids (mainly lactic acid and acetic acid) that lower the pH of the sourdough medium. In addition, these organisms are responsible for the production of ethanol, aroma compounds, bacteriocins, exopolysaccharides, and several enzymes (17). Sourdough LAB may originate with natural contaminants in the flour or with a starter culture which contains one or more LAB strains (6). Sourdough can be cultivated in bakeries or obtained from commercial suppliers. In Belgium, many artisan bakeries still use spontaneously fermented sourdoughs, which are kept metabolically active through the addition of flour and water at regular intervals (backslopping). During this process of continuous propagation, microbial associations with a remarkably high stability develop in the sourdough (5). However, the exact impact of process technology, the production environment, and many other factors on the composition and evolution of bacterial sourdough populations remains unclear. A recent study demonstrated that the LAB composition in traditional Belgian sourdoughs is influenced by the bakery environment rather than by the type of flour (28). Still, it remains unclear how these and other ecological factors influence the microbiological composition and metabolic characteristics of the final sourdough when temporal variability during continuous propagation also is taken into account (6, 15, 20). Clearly, a better knowledge of the parameters that may lead to variation among bacterial sourdough associations during the backslopping process will lead to better-controlled processes and standardization of high-quality baked goods.

In a previous study (28), biodiversity data from Belgian sourdough ecosystems were obtained through a conventional isolation strategy followed by molecular identification of selected isolates. The most obvious advantage of culture-based methods is that a well-documented collection of biological reference material is available for further in-depth taxonomic and metabolic analyses. On the other hand, this approach is labor intensive and lacks the broad coverage required to ana-

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lyze temporal variations in complex bacterial communities occurring in natural food ecosystems. Culture-independent methods, such as denaturing gradient gel electrophoresis of PCR amplicons (PCR-DGGE), are commonly used to circumvent the limitations of conventional cultivation (7). PCR-DGGE has the potential to characterize and monitor the microbial population involved in fermentation processes (24) and has been successfully applied to study the LAB composition and population dynamics of sourdough ecosystems (11, 20, 21, 26). In contrast to culturing, however, PCR-DGGE strategies visualize only the predominant members of a bacterial community and do not provide information at the individual strain level (7, 20, 23).

In the present study, culturing and PCR-DGGE population profiling were combined to examine the taxonomic structure and stability of Belgian artisan sourdoughs sampled twice at 11 geographically separated bakeries with a 1-year interval. In parallel, metabolite target analysis was performed in order to generate a composite data set in which the relation between the biodiversity data and sourdough fermentation profiles could be statistically analyzed.

MATERIALS AND METHODS

Strains and growth conditions. The LAB strains included in this study were cultured on MRS-5 medium, a modified de Man-Rogosa-Sharpe medium described by Meroth et al. (20). All LAB reference strains were obtained from the BCCM/LMG Bacteria Collection (Ghent University, Ghent, Belgium [http://bccm.belspo.be]).

Sourdough sampling, isolation of LAB, and phenotypic characterization. Eleven different bakeries throughout Belgium were selected for two sampling campaigns with traditional sourdoughs. During a first sampling campaign (SC1), from May to October 2004, a total of 21 sourdoughs were collected (28). Approximately 1 year later, the same bakeries were visited for a second sampling campaign (SC2), during which 18 sourdoughs were sampled. A uniform code was assigned to each sourdough sample encoding information on the depositor (D01 up to D11), the type of flour used to produce the sourdough (WW, wheat flour; RR, rye flour; SS, spelt flour; WR, mixture of wheat and rye flour; ME, mixture of wheat, rye and spelt flour), and the time of sampling (T). Collection and processing of samples, as well as sampling and microbiological and phenotypic analysis, were performed as described previously (28). Additionally, the pH, total titratable acidity (TTA), and dough yield of the sourdough samples were determined as described previously (31). Based on biodiversity data obtained from sourdoughs sampled during SC1 (28), the following minor adjustments were made to the protocol for microbiological analysis of samples collected during SC2. Dilutions of sourdough samples of SC2 were incubated aerobically and anaerobically at 30°C but not at 37°C. Based on differences in colony morphology, an average of 30 colonies per sourdough sample were selected from MRS-5 agar plates containing between 20 and 200 colonies. From these, only catalase-negative isolates were selected for further characterization and identification.

Molecular identification of sourdough LAB isolates. The selected isolates were subjected to repetitive DNA element PCR analysis. Genomic DNA was extracted, and $(GTG)_5$-PCR fingerprint analyses were carried out as described previously (28). The resulting fingerprints were analyzed using the BioNumerics v4.61 software package (Applied Maths, Sint-Martens-Latem, Belgium) and compared with an extensive in-house reference database. The similarity among the digitized profiles was calculated using the Pearson correlation coefficient, and an unweighted pair-group method using arithmetic averages (UPGMA) dendrogram was derived from the profiles. $(GTG)_5$-PCR fingerprinting was used to reduce the initial collection of LAB isolates to a set of genotypically unique isolates based on visual interpretation of band pattern complexity. For identification purposes, $(GTG)_5$-PCR clusters were delineated at 50% Pearson similarity and isolates were tentatively assigned to a given species when they belonged to a cluster that contained one or more type or reference strains. To verify the tentative identifications obtained by $(GTG)_5$-PCR clustering and to identify any remaining unknown isolates, one or more representatives of each cluster were subjected to a polyphasic taxonomic approach, including identification through *pheS* gene and 16S rRNA gene sequence analysis and/or DNA-DNA hybridizations (28). Newly obtained *pheS* gene sequences were imported into the BioNumerics v4.61 software program (Applied Maths), aligned, and compared using the neighbor-joining method with publicly available sequences of LAB type and reference strains. If the taxonomic position could not be revealed by *pheS* sequencing, representatives of each *pheS* cluster were characterized by 16S rRNA gene analysis and/or DNA-DNA hybridizations. Closest relatives of 16S rRNA gene sequences were determined by performing a search in GenBank using the BLAST algorithm.

Microbial stability of artisan sourdough samples analyzed through PCR-DGGE analysis. The procedures for DNA extraction from fresh sourdough samples and subsequent PCR-DGGE analysis were described previously (31). In addition, MRS-5 cultivable fractions obtained from sourdough samples collected during SC2 were subjected to PCR-DGGE. For this purpose, 50 μl of 10^{-1} serial dilutions of these sourdough samples was plated on MRS-5 agar. After incubation, the bacterial fraction grown on the MRS-5 agar plates was harvested for DNA extraction and subsequent DGGE analysis. In the present study, three different denaturing gradients were applied (i.e., 35 to 70%, 30 to 50%, and 50 to 70%). By inclusion of a reference pattern (31) every five lanes on each DGGE gel, resulting band profiles could be digitally normalized by comparison with a standard reference using the BioNumerics v4.61 software package. This normalization enabled comparison of migration distances between different DGGE gels. Purification and sequencing of DGGE bands was performed as described previously (31).

Metabolite target analysis. Concentrations of sugars, amino acids, sugar metabolites, and amino acid metabolites were determined with high-performance liquid chromatography, high performance anion exchange chromatography coupled to mass spectrometry, and gas chromatography coupled to mass spectrometry as described previously (31). Volatiles were measured with gas chromatography coupled to mass spectrometry through static headspace analysis (31). All samples were analyzed in triplicate, and the mean values ± standard deviations are represented, except for the results of the headspace analysis, which were expressed as [100 × peak area compound (peak area internal standard × g sample)$^{-1}$].

Data analysis. All data processing and statistical analysis were performed using the software package BioNumerics v4.61. For each sourdough sample, a microbial community profile was composed that reflects the qualitative (number of isolated species) and quantitative (number of isolates per species, expressed as percentage values) diversity of sourdough LAB in the corresponding sample (28). Similarities among community profiles were expressed using the Pearson product-moment correlation coefficient. For sample comparison based on PCR-DGGE data, the band-based Dice coefficient was used. In addition, a band-matching analysis was carried out and DGGE bands were assigned to classes of common bands within all sample profiles. Due to the complexity of several DGGE profiles, two denaturing gradients (i.e., 30 to 50% and 50 to 70%) were used to properly assign bands to classes. Subsequently, a composite data set was generated from the culture-dependent and culture-independent data sets. To visualize similarities among the microbial sourdough communities, a transversal cluster analysis (two-way clustering) was performed to associate groups of characters (bacterial species and DGGE bands) with groups of sourdough samples. Similarity values between sourdough samples were calculated using the average of the similarity matrices obtained for diversity and DGGE band-matching data. For clustering of the characters, a UPGMA dendrogram was constructed and similarities were expressed using the Pearson product-moment correlation coefficient. Prior to statistical analysis, the relative concentrations of the volatile compounds were converted into binary data (0/1 if volatile was absent/present). Subsequently, these data were merged with sugar, amino acid, and metabolite concentrations for the 39 sourdough samples. From these combined metabolic data, a consensus matrix was calculated using the values from the similarities from each individual data set and a UPGMA dendrogram was constructed. For clustering of the metabolic characters, similarities were expressed using the Pearson product-moment correlation coefficient. In addition, a principal component analysis (PCA) was performed on the composite data set of the culture-dependent, culture-independent, and metabolic data to visualize relationships and to investigate the contribution of these parameters to the variation of the data using the BioNumerics software.

Nucleotide sequence accession numbers. The EMBL accession numbers for newly determined sequences of the LAB isolates selected for *pheS* gene sequencing are AM901454 to AM901541 (SC2).

RESULTS

Sourdough sampling and enumeration of LAB and yeasts. A total of 39 traditional Belgian sourdoughs, all produced without the addition of a commercial starter or yeast, were sampled in 11 bakeries throughout Belgium. These sourdoughs differed in one

TABLE 1. General characteristics and culture-based LAB composition of 39 Belgian sourdoughs sampled during the first and second sampling campaigns

Dep.[a]	Sample code	Flour type[b]	SC[c]	Province[d]	Age[e] (yr)	pH[f]	Time[g] (h)	Temp[h]	LAB/yeast ratio	Species identified by culture-dependent analysis[i]
D01	D01WW01T01	WW	1	E-F	5	3.83	10	30	1.72E + 02	*L. crustorum* (2/40), *L. helveticus* (6/40), *L. paralimentarius* (9/40), *L. plantarum* (5/40), *L. pontis* (9/40), *L. rossiae* (1/40), *L. spicheri* (8/40)
	D01WW01T02	WW	2	E-F	6	3.54	70	30	1.37E + 01	*L. crustorum* (6/33), *L. helveticus* (3/33), *L. paralimentarius* (11/33), *L. plantarum* (4/33), *L. pontis* (2/33), *L. spicheri* (7/33)
	D01WW01T03	WW	2	E-F	6	3.65	9	30	2.25E + 01	*L. crustorum* (1/34), *L. helveticus* (5/34), *L. paracasei* (1/34), *L. paralimentarius* (9/34), *L. plantarum* (5/34), *L. pontis* (6/34), *L. spicheri* (7/34)
D02	D02WR01T01	WR	1	W-F	25	4.01	<24	AT	7.44E + 04	*L. curvatus* (10/31), *L. plantarum* (3/31), *L. sanfranciscensis* (18/31)
	D02WR01T02	WR	1	W-F	25	3.80	22	AT	1.16E + 05	*L. sanfranciscensis* (26/26)
	D02WR01T03	WR	2	W-F	26	3.66	12	AT	1.91E + 05	*L. sanfranciscensis* (29/29)
	D02WW01T01	WW	1	W-F	2	3.83	22	AT	2.87E + 03	*L. sakei* (1/29), *L. sanfranciscensis* (28/29)
	D02WW01T02	WW	2	W-F	3	4.45	26	AT	2.02E + 03	*L. sanfranciscensis* (28/28)
D03	D03WW01T01	WW	1	W-F	6	4.12	4	30	2.67E + 01	*L. paralimentarius* (22/40), *L. pontis* (12/40), *L. rossiae* (6/40)
	D03WW01T02	WW	2	W-F	7	3.50	30	AT	2.30E + 01	*L. paralimentarius* (2/18), *L. pontis* (10/18), *L. rossiae* (6/18)
D04	D04WW01T01	WW	1	BW	1	5.00	0	AT	2.27E + 05	*L. sakei* (24/38), *L. sanfranciscensis* (12/38), *L. mesenteroides* (2/38)
	D04WW01T02	WW	2	BW	2	4.13	1	AT	5.64E + 03	*L. sanfranciscensis* (12/12)
	D04WW01T03	WW	2	BW	2	4.51	0	AT	5.69E + 06	*L. sanfranciscensis* (12/12)
D05	D05WW01T01	WW	1	E-F	1	3.63	24	28	5.20E + 01	*L. crustorum* (1/40), *L. helveticus* (9/40), *L. paracasei* (1/40), *L. pontis* (14/40), *L. sanfranciscensis* (15/40)
	D05WW01T02	WW	2	E-F	2	3.57	25	28	2.00E + 01	*L. helveticus* (1/28), *L. pontis* (12/28), *L. sanfranciscensis* (15/28)
D06	D06SS01T01	SS	1	L	1.5	5.04	2	21–23	1.41E + 03	*L. curvatus* (1/40), *L. plantarum* (10/40), *L. sakei* (1/40), *P. pentosaceus* (2/40), *W. cibaria* (26/40)
	D06SS01T02	SS	2	L	2.5	4.45	2	21–23	9.35E + 03	*L. sanfranciscensis* (30/30)
	D06WW01T01	WW	1	L	12	4.75	2	21–23	8.97E + 02	*L. paralimentarius* (2/41), *L. plantarum* (12/41), *L. sanfranciscensis* (7/41), *P. pentosaceus* (3/41), *W. cibaria* (17/41)
D07	D07WR01T01	WR	1	H	0.8	3.83	13	26	1.23E + 01	*L. brevis* (1/41), *L. fermentum* (14/41), *L. paralimentarius* (9/41), *L. pontis* (15/41), *P. pentosaceus* (2/41)
	D07WR01T02	WR	1	H	0.8	3.86	13	26	4.98E + 00	*E. mundtii* (1/40), *L. brevis* (1/40), *L. fermentum* (14/40), *L. paralimentarius* (16/40), *L. pontis* (5/40), *W. confusa* (2/40)
	D07WR01T03	WR	2	H	2	3.64	42	26	1.40E + 01	*L. brevis* (4/23), *L. paracasei* (1/23), *L. paralimentarius* (14/23), *L. rossiae* (3/23), *P. acidilactici* (3/23)
	D07WR02T01	WR	1	H	0.01	3.78	12	AT	8.17E + 01	*L. fermentum* (33/39), *L. pontis* (3/39), *P. acidilactici* (3/39)

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TABLE 1—*Continued*

Dep.[a]	Sample code	Flour type[b]	SC[c]	Province[d]	Age[e] (yr)	pH[f]	Time[g] (h)	Temp[h]	LAB/yeast ratio	Species identified by culture-dependent analysis[i]
D08	D08WW01T01	WW	1	N	15	3.80	6	AT	1.86E + 02	*L. buchneri* (1/40), *L. parabuchneri* (8/40), *L. plantarum* (27/40), *L. rossiae* (4/40)
	D08SS01T01	SS	2	N	16	3.76	8	AT	2.80E + 01	*L. plantarum* (1/29), *L. sanfranciscensis* (28/29)
D09	D09ME01T01	ME	1	N	4	3.78	<24	AT	1.40E + 01	*L. brevis* (1/40), *L. hammesii* (14/40), *L. namurensis* (6/40), *L. paralimentarius* (19/40)
	D09ME01T02	ME	2	N	5	3.72	12	AT	1.50E + 01	*L. brevis* (1/30), *L. hammesii* (7/30), *L. namurensis* (3/30), *L. paralimentarius* (18/30), *L. plantarum* (1/30)
D10	D10SS01T01	SS	1	Lux	3	4.28	4	28	4.92E + 02	*L. pontis* (5/15), *L. sanfranciscensis* (9/15)
	D10SS01T02	SS	2	Lux	4	4.74	1	28	1.19E + 05	*L. sanfranciscensis* (30/30)
	D10WR01T01	WR	1	Lux	3	3.96	4	28	3.28E + 02	*L. pontis* (3/9), *L. sanfranciscensis* (4/7)
	D10WW01T01	WW	1	Lux	3	4.09	4	28	4.53E + 02	*L. pontis* (4/9), *L. sanfranciscensis* (4/9), *P. pentosaceus* (1/9)
	D10WW01T02	WW	2	Lux	4	4.78	1	28	5.10E + 03	*L. sanfranciscensis* (30/30)
D11	D11RR01T01	RR	1	N	2	3.79	12	AT	4.17E + 01	*L. hammesii* (11/38), *L. paralimentarius* (26/38), *L. plantarum* (1/38)
	D11RR01T02	RR	2	N	3	4.59	12.5	AT	2.11E + 01	*L. brevis* (1/29), *L. hammesii* (7/29), *L. nantensis* (16/29), *L. plantarum* (5/29)
	D11SS01T01	SS	1	N	2	3.83	12	AT	5.38E + 01	*L. brevis* (5/40), *L. hammesii* (12/40), *L. nantensis* (1/40), *L. paralimentarius* (15/40), *L. plantarum* (7/40)
	D11SS01T02	SS	2	N	3	4.16	12.5	AT	1.90E + 01	*L. brevis* (4/30), *L. hammesii* (5/30), *L. nantensis* (16/30), *L. plantarum* (4/30), *L. rossiae* (1/30)
	D11WW01T01	WW	1	N	2	3.72	12	AT	7.23E + 01	*L. brevis* (7/40), *L. hammesii* (8/40), *L. nantensis* (4/40), *L. paralimentarius* (13/40), *L. plantarum* (8/40)
	D11WW01T02	WW	2	N	3	4.41	12.5	AT	2.29E + 01	*L. brevis* (3/29), *L. hammesii* (4/29), *L. nantensis* (11/29), *L. plantarum* (10/29), *W. confusa* (1/29)
	D11WW02T01	WW	1	N	2	3.85	12	AT	3.73E + 01	*L. hammesii* (14/40), *L. nantensis* (2/40), *L. paralimentarius* (20/40), *L. plantarum* (4/40)
	D11WW02T02	WW	2	N	3	4.17	12.5	AT	3.27E + 01	*L. brevis* (5/26), *L. hammesii* (4/26), *L. nantensis* (10/26), *L. plantarum* (7/26)

[a] Depositor.
[b] Type of flour: WW, wheat; SS, spelt; RR, rye; WR, wheat-rye; ME, wheat-rye-spelt.
[c] Sampling campaign 1 or 2.
[d] Province: E-F, East-Flanders; W-F, West-Flanders; BW, Brabant Wallon; L, Liège; H, Hainaut; N, Namur; Lux, Luxembourg.
[e] Age of the mother dough in years.
[f] Each value is the average from three independent analyses, and the standard deviation was always less than 5%.
[g] Fermentation time.
[h] Fermentation temperature; AT, ambient temperature.
[i] *P. pentosaceus, Pediococcus pentosaceus; W. cibaria, Weissella cibaria; W. confusa, Weissella confusa; E. mundtii, Enterococcus mundtii; P. acidilactici, Pediococcus acidilactici; L. mesenteroides, Leuconostoc mesenteroides;* all others belong to the genus *Lactobacillus.* For each sample, the number of isolates of each species relative to the total number of identified isolates is indicated in parentheses.

or more process parameters, such as fermentation temperature, fermentation time, pH, and age (Table 1). Several sourdoughs had been sustained for several years, whereas others were recently initiated. Generally, a high pH and low TTA value were observed when samples were taken shortly after backslopping. A low pH and high TTA value were observed for sourdoughs with longer incubation times. Microbiological analysis showed that LAB constitute the major microbiota of the sourdoughs. LAB

counts on MRS-5 agar ranged from 10^7 to 10^9 CFU/g sourdough. For yeasts, counts ranged from 10^2 to 10^7 CFU/g sourdough on yeast glucose agar. In general, the LAB and yeast counts for samples originating from SC1 were similar to those for SC2 (data not shown). Consequently, comparison of the LAB/yeast ratios of bakery samples collected during both sampling campaigns yielded highly similar values, except for samples D01WW01T01/02, D04WW01T01/02/03, D10SS01T01/02, and D10WW01T01/02, where the difference in LAB/yeast ratio between both sampling campaigns exceeded 1 log unit (Table 1).

LAB diversity of artisan sourdoughs through cultivation. In total, 1,194 potential LAB isolates were selected from MRS-5 agar plates, of which 714 were collected during SC1 and 480 during SC2. Numerical analysis of digitized $(GTG)_5$-PCR fingerprints with the reference database resulted in the delineation of 24 and 17 different clusters among the isolates of the SC1 (28) and SC2 subsets, respectively. This allowed tentative identification of 514 LAB isolates collected during SC1 (72% of total) and 465 LAB isolates (97% of total) collected during SC2, belonging to 10 and 13 different $(GTG)_5$-PCR clusters, respectively. Subsequently, one or more representative strains from each $(GTG)_5$-PCR cluster were selected and identified by *pheS* gene sequence analysis and/or 16S rRNA gene sequencing followed by DNA-DNA hybridizations (28). This approach allowed identification of all remaining isolates (Table 1). Subsequent to polyphasic identification of the sourdough LAB strains collected during SC1, the corresponding fingerprint patterns were included in the $(GTG)_5$-PCR reference framework. As a result, a higher number of isolates (97%) collected during SC2 could be identified by $(GTG)_5$-PCR fingerprint analysis than was the case for those from SC1 (72%).

The (facultatively) heterofermentative species *Lactobacillus paralimentarius*, *Lactobacillus plantarum*, *Lactobacillus pontis*, and *Lactobacillus sanfranciscensis* were the most frequently isolated taxa and occurred in 15, 16, 13, and 18 sourdough samples, respectively (Table 1). In most bakeries, the qualitative and quantitative LAB diversity observed in the traditional sourdoughs is relatively similar for the two sampling rounds. In bakeries D04, D06, D07, D08, and D11, however, variations in the composition of the dominating LAB species were observed. Sourdough samples produced at bakeries D04, D06, D07, and D08 during SC2 showed a lower degree of diversity than the corresponding sourdoughs produced during SC1. Although the numbers of species found in sourdoughs produced by depositor 11 were comparable over the two sampling campaigns, *L. paralimentarius* was not isolated from samples produced during SC2 whereas this species was dominant in D11 sourdoughs sampled during SC1. While *L. paralimentarius* appeared to be the most frequently isolated species in sourdoughs produced during SC1, *L. sanfranciscensis* prevailed during SC2.

Interpretation of PCR-DGGE population fingerprints. The microbial stability of sourdoughs sampled during SC1 and SC2 was studied by using PCR-DGGE of V3-16S rRNA gene amplicons (Fig. 1). In addition to the study of sourdough LAB community profiles, a selection of identified sourdough isolates was also subjected to PCR-DGGE analysis. Strains of about half of the isolated LAB species (46%) produced a single DGGE band, whereas strains from the remaining species showed multiple bands due to operon heterogeneity. For eight species (33%), including *L. brevis*, *L. fermentum*, *L. helveticus*, *L. paracasei*, *L. paralimentarius*, *L. plantarum*, *L. pontis*, and *L. sanfranciscensis*, genotypically different strains of the same species produced different DGGE profiles. Moreover, comigration of V3-16S rRNA gene amplicons of two or more species was observed. For instance, comigration of *L. curvatus*, *L. sakei*, and *L. helveticus* in a 35 to 70% denaturing gradient gel was observed, which may partly be explained by the close phylogenetic relatedness between *L. curvatus* and *L. sakei* (99.5% 16S rRNA gene similarity). Because the effects of intraspecific and operon heterogeneity and comigration of DGGE bands hampered the immediate assessment of the predominant species diversity using DGGE community profiles, the initial characterization of the individual DGGE bands mainly relied on comparison of band positions with those of purified sourdough isolates.

For sourdough samples of SC2, the cultivable fraction of the LAB population also was analyzed by PCR-DGGE. This approach appeared to be a useful addition to PCR-DGGE analysis of total sourdough DNA extracts, since additional bands were observed in the profiles from MRS-5 cultivable fractions. In all cases, these bands could be assigned to species which were also recovered on MRS-5 agar. In fact, in some sourdough samples a number of species were recovered by culturing that could not be detected by PCR-DGGE. Given the number of isolates recovered, however, these species probably represent only a minority of the total bacterial diversity in Belgian sourdoughs. On the other hand, a number of bands in the sourdough fingerprints did not correspond to any of the species identified through band positioning of pure cultures. These bands were extracted and sequenced, and the resulting partial rRNA gene sequences (160 bp) were compared by BLAST analysis with the EMBL database (31). For example, the faint high %GC DGGE band A in Fig. 1 which was detected in the DGGE profiles of sourdough samples D01WW01T01 and D07WR01T03 could be assigned to an *Acetobacter* species (96%). Furthermore, PCR-DGGE allowed the detection of a member of the *Erwinia*/*Enterobacter*/*Pantoea* group in samples D07WR01T01/02 (DGGE band D in Fig. 1).

Through extraction and sequencing, it was found that a number of bands were PCR artifacts that did not represent 16S rRNA gene templates occurring in the sample. These were the result of heteroduplex formation between highly similar but nonidentical sequences during a mixed-template PCR (24). Excision and reamplification of heteroduplex bands yielded both the original and the heteroduplex products that migrate higher in the DGGE gradient as a result of one or more base pair mismatches. For example, reamplification of band BC′ (Fig. 1) yielded three different products that migrated to positions corresponding to band BC′ (heteroduplex product), band BC (*L. hammesii*), and band B′C′ (*L. brevis*). Theoretically, one would expect two heteroduplex bands in the gel (BC′ and B′C) (24). However, since no second heteroduplex band was detected in the DGGE profile of the corresponding sourdough sample, the two heteroduplex molecules probably migrated at the same position in the gradient gel. Sequencing of the original pure amplicons and heteroduplex amplicons revealed base pair differences at specific positions in the component sequences and double-peak profiles at the same positions in the heteroduplex sequence (data not shown). Although *L. panis* was not isolated through culture-dependent analysis, a

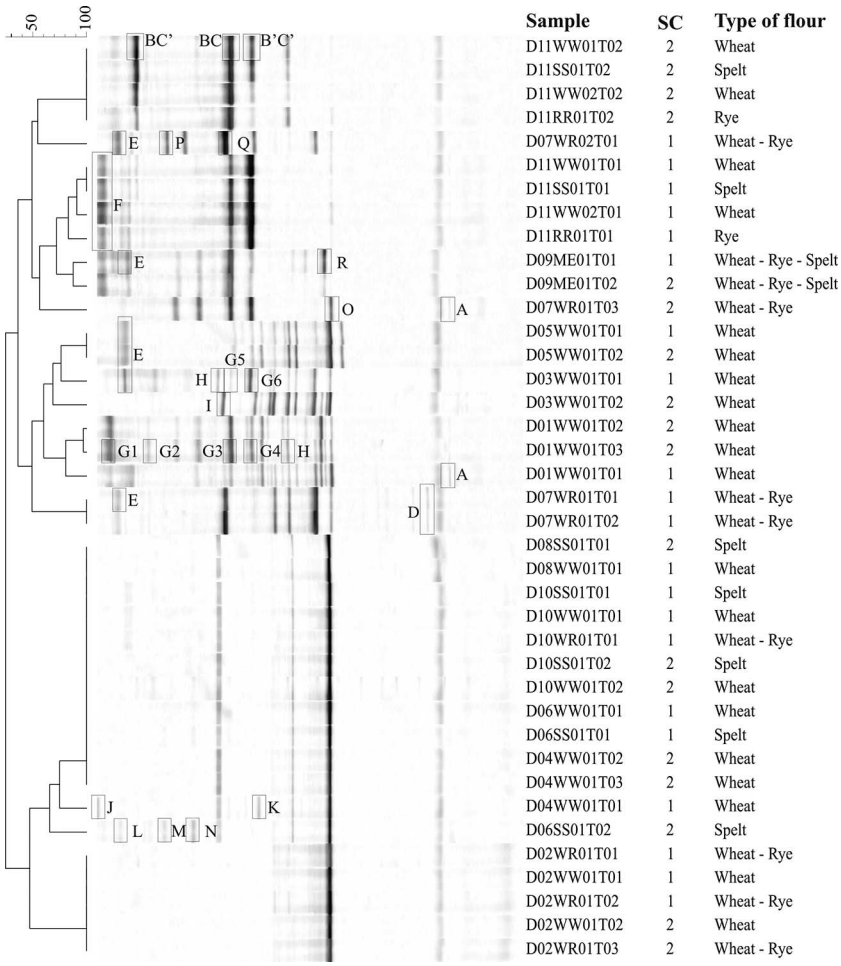

FIG. 1. Dendrogram showing similarities among band profiles from PCR-DGGE analysis of 16S rRNA gene amplicons of 39 traditional Belgian sourdoughs using Dice coefficient and UPGMA. Most bands were characterized by comparing the DGGE band positions with those of pure cultures isolated from the corresponding sourdough sample. The remaining undefined or unclear bands were characterized by 16S rRNA gene sequencing (bands A to R). Band A, *Acetobacter* sp.; band BC′, heteroduplex *L. hammesii*/*L. brevis*; band BC, *L. hammesii*; band B′C′, *L. brevis*; band D, member of the *Erwinia*/*Enterobacter*/*Pantoea* group; band E, member of the *L. reuteri* group (>97% sequence similarity with *L. pontis*/*L. panis*/*L. gastricus*/*L. coleohominis*); band F, unidentified (low sequence similarity with members of the *L. buchneri* group); bands G1 to -6, member of the *L. plantarum* group (>97% sequence similarity with *L. alimentarius*/*L. paralimentarius*/*L. kimchii*/*L. crustorum*/*L. farciminis*/*L. mindensis*); band H, *L. rossiae*; band I, heteroduplex *L. pontis*/*L. panis*; band J, heteroduplex *L. sakei*/*L. sanfranciscensis*; band K, *L. sakei*; band L, *L. plantarum*; bands M and N, *Weissella cibaria* or *Weissella confusa*; band O, mitochondrial cereal DNA; bands P and Q, *L. fermentum*; band R, *L. namurensis.* SC, sampling campaign.

heteroduplex molecule of *L. panis* and *L. pontis* was detected through PCR-DGGE analysis (Fig. 1, band I), which revealed for the first time the presence of *L. panis* in Belgian sourdoughs. Several band positions in the DGGE fingerprints of D03WW01T01 and D01WW01T03 could be assigned to the *L. plantarum* group based on band sequencing (bands G1 to G6; Fig. 1). However, band position analysis with type and reference strains of *L. plantarum* group members, namely, *L. plantarum*, *L. alimentarius*, *L. paralimentarius*, *L. kimchii*, *L. farciminis*, *L. crustorum*, *L. mindensis*, and *L. nantensis*, did not allow the further assignment of any of these bands to a specific species. Possibly, these bands correspond to a currently undescribed *Lactobacillus* species that was not retrieved on MRS-5 agar. Similarly, band E was tentatively assigned to a member of the *L. reuteri* group by sequencing but could not be linked to a known species in this group (Fig. 1).

Microbial stability of artisan sourdoughs based on PCR-DGGE and cultivation. Although the relative intensities of dominant bands could vary among sourdoughs produced within one bakery, cluster analysis of digitized DGGE profiles revealed that sourdoughs produced in the same bakery had a similar dominant microbial composition, irrespective of the period of sampling (Fig. 1). As an exception, the DGGE profiles of sourdoughs produced by depositor D07, using different wheat-rye flour compositions or sampled on different occasions, demonstrated a remarkable difference in microbial diversity. This finding is in correspondence with the differences in LAB diversity obtained by culture-dependent analysis (Table 1).

Transversal cluster analysis of the composite data set from culture-dependent and -independent approaches associated groups of samples and characters (identified species and band

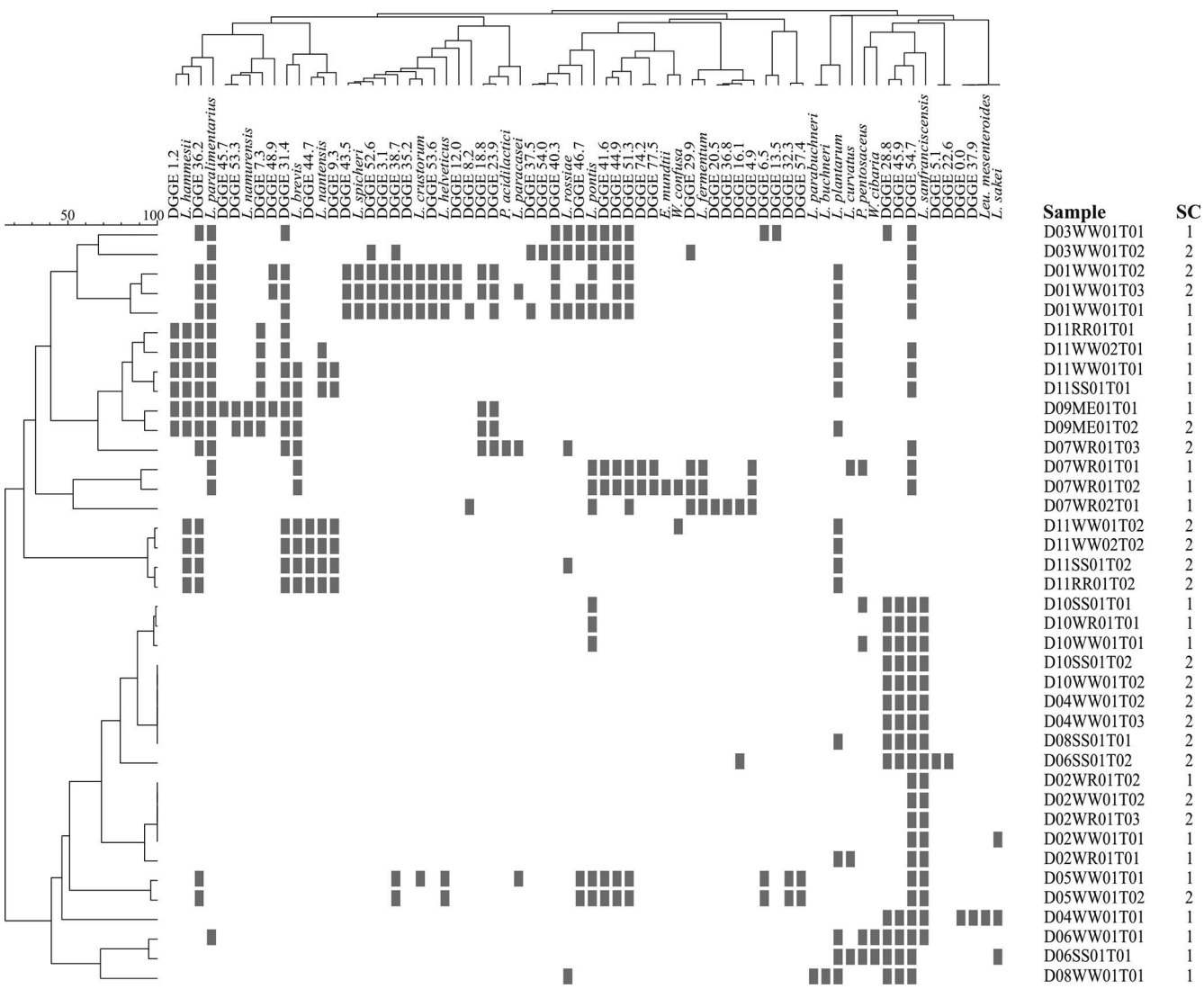

FIG. 2. Transversal dendrogram showing the similarities among 39 traditional sourdough samples. Analysis was based on a combined data set, reflecting the diversity of LAB species isolated from 39 sourdough samples and 44 band classes representing common bands in the corresponding sourdough DGGE fingerprints. Similarities between sourdough samples were expressed as percent similarity values and represent the average of diversity and DGGE band-matching similarity matrices. To visualize similarities between character data (bacterial species and DGGE bands), an UPGMA dendrogram was constructed using the Pearson product-moment correlation coefficient.

classes) with a high degree of correlation (Fig. 2). For example, cluster analysis of the characters associated the frequently isolated species *L. sanfranciscensis* with DGGE band classes 28.8, 45.9, and 54.7, all of which corresponded to different operons of the 16S rRNA gene of *L. sanfranciscensis* LMG 16002^T. This cluster analysis appeared to confirm the overall stability of LAB communities between the two sampling campaigns (Fig. 2), although some salient discrepancies were observed for samples originating from depositors D04, D06, D07, D08, and D11. Variations in the technological parameters applied to produce sourdoughs during the two sampling campaigns might explain some of these discrepancies, but such differences were observed only for sourdoughs produced by depositor 7 (Table 2). Samples D07WR01T01/T02 and D07WR01T03 differ in flour composition, age of the dough, fermentation, and refrigeration conditions, and each of these technological parameters may contribute to the observed differences in bacterial diversity (Fig. 2). In addition, depositor 7 modified the sourdough fermentation protocol in the course of this study by supplementing the sourdoughs produced in SC2 with fructose. Possibly, this modification contributed to the striking difference in the microbial composition between samples produced in SC1 and SC2.

In the case of the sourdoughs originating from depositors 4, 6, 8, and 11, presumed indications of instability between both sampling campaigns could mostly be linked to differences in detection capacity intrinsic to the combined use of culturing and PCR-DGGE. For instance, in samples produced by depositor 11, *L. paralimentarius* was isolated during SC1 but not during SC2, yet faint bands corresponding to *L. paralimentarius* were observed in the DGGE profiles of sourdoughs sampled during SC2. However, since V3 amplicons of *L. paralimentarius*

TABLE 2. Technological characteristics of sourdoughs produced by depositor 7

Sample code	Sampling campaign	Flour composition	Age of sourdough	Fermentation conditions	Refrigeration before sampling
D07WR01T01	1	1/6 wheat + 5/6 rye	10 mo	13 h, 26°C	80 h, 6°C
D07WR01T02	1	1/6 wheat + 5/6 rye	10 mo	13 h, 26°C	11 h, 6°C
D07WR02T01	1	2/3 wheat + 1/3 rye	60 h	12 h, ambient temp	None
D07WR01T03	2	1/6 wheat + 5/6 rye	2 yr	16 h, 26°C	26 h, 6°C

and *L. brevis* have identical melting positions in the denaturant gradient and *L. brevis* was isolated from samples of both sampling campaigns, DGGE cannot elucidate if *L. paralimentarius* either was not present in the sourdough samples produced during SC2 or was not selected for cultivation and subsequent identification. In contrast, band class 44.7, which was assigned to *L. nantensis*, was detected only in sourdough profiles of SC2. However, a few isolates of *L. nantensis* were recovered from three of the four samples produced during SC1, suggesting that this species was present in low concentrations and undetectable by PCR-DGGE.

Metabolite target analysis. The main sugars found in most sourdoughs were maltose (up to 34.44 g kg^{-1}), glucose (up to 9.14 g kg^{-1}), and fructose (up to 2.66 g kg^{-1}), but lower concentrations of arabinose (up to 1.17 g kg^{-1}), sucrose (up to 1.09 g kg^{-1}), and xylose (up to 0.14 g kg^{-1}) were detected too. The most important sugar metabolites were lactic acid (up to 13.71 g kg^{-1}), acetic acid (up to 1.74 g kg^{-1}), ethanol (up to 24.38 g kg^{-1}), and mannitol (up to 7.32 g kg^{-1}). Succinic acid and erythritol were found in low concentrations (below 0.65 g kg^{-1}). Concerning the metabolites of arginine (0.00 to 0.99 mmol kg^{-1}) conversion, ornithine was found in many sourdough samples in concentrations lower than 1 ± 0.1 mmol kg^{-1}, whereas citrulline was hardly detected. Metabolites of aromatic amino acids, mostly hydroxy acids, were often encountered in the sourdough samples. The GC headspace analysis revealed that ethanol and ethylacetate were the most important volatiles. Other common volatiles included 2-methyl-1-propanol, 3-methyl-1-butanol, and 1-propanol. Also, aldehydes, such as hexanal and acetaldehyde, and esters, such as 2-hydroxypropanoic acid propyl ester and 3-methylbutanol acetate, were found. To study the metabolic stability of Belgian sourdough ecosystems, all metabolic data (sugars, sugar metabolites, amino acids, arginine metabolites, aromatic amino acid metabolites, and volatiles) for the 39 sourdough samples were merged in a composite data set for transversal cluster analysis (see Fig. S1 in the supplemental material). In contrast to transversal cluster analysis of the taxonomic data, sourdough metabolic profiles did not consistently group samples according to bakery origin and in fact shared many metabolic characteristics across all samples. Probably due to the metabolic adaptation of most typical sourdough LAB to the carbohydrate and protein sources available in sourdoughs, the large majority of sugar and amino acid metabolites were detected in all sourdough samples, irrespective of their LAB biodiversity. Yet concentrations of a number of individual metabolic compounds varied considerably among samples, which might be explained by variations in technological parameters (e.g., time of fermentation) between samples or by strain-specific metabolic properties within a given species. Although variations in the metabolic profiles were mainly attributed to volatile, aromatic amino acid and arginine metabolic products, no correlation between sourdough LAB and metabolic compounds was found.

PCA-based analysis of microbial and metabolic stability of traditional Belgian sourdoughs. To study the overall stability of Belgian sourdough ecosystems, all data obtained for the 39 sourdough samples (i.e., diversity of isolated LAB species, DGGE band matching table, concentrations of sugars and amino acids, metabolite concentrations, and the occurrence of volatiles) were merged in a composite data set for PCA analysis. Although many bakeries produced different types of sourdoughs from different flour types and each sourdough was sampled during two sampling campaigns, PCA revealed only limited variation among the different sourdough samples originating from a single bakery (see Fig. S2 in the supplemental material).

The first five principal components accounted for about 85% of the variation and corresponded to the isolation of *L. sanfranciscensis* on MRS-5, the detection of DGGE bands 45.9 and 28.8 (both assigned to *L. sanfranciscensis*), and the presence of arginine and sucrose, respectively. The occurrence of *L. sanfransciscensis* (detected by cultivation and DGGE) was positively correlated with sourdough samples originating from depositors D02, D04, D06, D08, and D10. Moreover, these samples clearly grouped separately from the other samples. Arginine production was negatively correlated with samples produced by depositors D01, D03, and D05, whereas the occurrence of sucrose was positively correlated with sourdoughs of depositors D04, D06, and D10. The highest variability among samples within a single bakery was observed for samples originating from depositor D07 and originated from differences in microbial biodiversity (analyzed through cultivation and PCR-DGGE) and in metabolite composition. For this depositor, the volatile component 1-butanol-3-methylacetate was absent in the sample of SC2 but present in all samples of SC1. Although the LAB diversity and DGGE profiles of samples originating from depositor D11 indicate a microbial composition that was not stable over time, we observed that the metabolite composition of the sourdough samples was considerably similar (see Fig. S1 in the supplemental material). Consequently, sourdough samples produced by depositor D11 grouped more closely together in PCA analysis based on both taxonomic and metabolic data. On the contrary, sourdough DNA profiles and LAB diversity data of samples produced by depositor D03 revealed a similar LAB composition, while PCA analysis revealed quite a bit of variation among the samples. Possibly, the different fermentation times used by this depositor to produce the sourdoughs (4 h versus 30 h; Table 1) resulted in different quantitative distributions of the LAB species, thus inducing different metabolic activities in the samples.

DISCUSSION

To develop new process technologies for the production of high-quality bakery products, a profound knowledge of factors determining microbial metabolism and stability of the involved microbiota involved is indispensable. Although the dynamics of total bacterial communities during sourdough fermentation were monitored previously (11, 20, 22, 31), only a few data are available on the long-term stability of sourdough ecosystems (2, 27). This study revealed that the microbial communities in the majority of the traditional Belgian sourdoughs varied little over time with respect to the number of microorganisms and the microbial diversity. As suggested before (28), the microbial community structure of Belgian traditional sourdoughs was mainly influenced by the bakery environment rather than the type of flour used to produce the sourdough. Still, there were indications that the species composition and metabolic activity in sourdoughs can be largely influenced by several process parameters (e.g., through the addition of fructose). Although not investigated in the present study, fermentation temperature also may have an impact on the competitiveness of lactobacilli in sourdough ecosystems (20). Differences in fermentation time do not appear to influence the microbial composition of sourdoughs but may affect the quantitative distribution of individual LAB species, as demonstrated for the sourdough samples produced by depositor D03.

Biodiversity data obtained through a cultivation-dependent isolation and identification approach did not fully correspond to the molecular inventory of sourdough samples through DGGE community fingerprinting. Species present in low concentrations may occasionally be picked up from MRS-5 agar plates but in many cases will not produce a detectable DGGE band in the sourdough DNA fingerprint. This finding illustrates the intrinsic limitation of DGGE analysis in visualizing only the predominant species of a microbial community (24). Although the random selection of colonies from MRS-5 agar plates enables isolation of less-dominant species, the inability of this medium to equally support growth of all species present in the sourdough samples can bias our understanding of the microbial sourdough diversity. For example, even though the type strain of *L. panis* is able to grow on MRS-5 agar (20) and was detected by 16S rRNA gene analysis of heteroduplex amplicons, this species was not isolated from any of the Belgian sourdoughs analyzed. This experimental bias, linked to the use of culture methods, demonstrates the need to simultaneously include culture-independent methods to study the microbial diversity of food fermentation processes (1, 6, 8, 12, 14, 18, 25, 26). Although DGGE analysis of cultivable bulk fractions remains a valuable option, this approach did not reveal the same degree of taxonomic diversity as the culture-dependent approach. In this context, the use of several media that may better reflect the complete diversity of a food sample could provide more-precise information about the concentration of the constituting species (8). Although the culture-dependent approach may be labor intensive and time consuming, the culture-independent PCR-DGGE method is also limited by some inherent biases. Among others, the occurrence of different melting positions in the denaturant gradient of strains belonging to the same species, 16S rRNA gene operon heterogeneity resulting in multiple DGGE bands for a single species, and the formation of heteroduplex molecules are all factors that may interfere with the interpretation of DGGE fingerprints and thus potentially lead to an overestimation of microbial diversity (24). In addition, this study demonstrated that different LAB sourdough species may yield V3-16S rRNA gene amplicons with 100% sequence similarity that have identical melting positions in the denaturing gradient. The use of primers targeting a single-copy housekeeping gene (3), other regions within the 16S rRNA gene (e.g., V6-V8 region of the 16S rRNA gene) (9), and/or the use of group-specific primers (19, 32) may circumvent some of these limitations. Additionally, biases may be introduced by actions preceding the actual DGGE analysis, such as nucleic acid extraction efficiency and selective amplification of 16S rRNA genes (21, 29). Considering specific limitations of both cultivation-independent and cultivation-dependent methods, a polyphasic approach for broad-coverage biodiversity studies of complex ecosystems should be recommended.

All Belgian sourdough samples analyzed in the present study were type I sourdoughs, which are often associated with the occurrence of *L. sanfranciscensis* as the dominating species (5, 26, 33). In about half of the sourdough samples, *L. sanfranciscensis* was detected by DGGE. Moreover, this species accounted for one-third of the LAB isolates. To some extent, the stable persistence of *L. sanfranciscensis* in sourdough ecosystems may be explained by its optimal growth temperature and pH values, which match the sourdough fermentation conditions (5, 10). In addition, some strains of *L. sanfranciscensis* are able to produce compounds with an antagonistic activity against other sourdough microorganisms (4, 13). In general, sourdough samples dominated by *L. sanfranciscensis* were characterized by high sucrose levels (up to 1.09 g/kg), and it has been reported that the majority of *L. sanfranciscensis* strains are not able to hydrolyze sucrose due to the lack of fructosyltransferase activity (30). Besides *L. sanfranciscensis*, the LAB species *L. paralimentarius*, *L. plantarum*, and *L. pontis* appear to dominate the LAB population of the sourdough samples analyzed in this study. These species reflect the type I sourdough microbiota (5), but to what extent these four species are typical for Belgian traditional sourdoughs remains unclear. In addition, it should be noted that a member of the *Erwinia*/*Enterobacter*/*Pantoea* group was detected in Belgian sourdoughs. Most probably, this organism originates from the flour used for sourdough production, given the fact that enterobacteria such as *Enterobacter cowanii* and *Pantoea agglomerans* have previously been isolated from nonsterilized flour (16). The occurrence of acetic acid bacteria, such as *Acetobacter*, in wine, vinegar, and cocoa fermentations is well documented, but to our knowledge, this is the first study to report the presence of *Acetobacter* species in sourdough.

In conclusion, this work highlights the need to combine both culture-dependent and culture-independent methods for a better description of complex microbial populations involved in the production of fermented foods, such as sourdough. Despite the use of different flour batches and possible variations in flour characteristics during subsequent propagation of the sourdoughs analyzed, the applied polyphasic approach revealed little temporal microbial and metabolic variation in Belgian traditional sourdough processes. In future studies, molecular strain typing could be applied to investigate whether this remarkable stability is linked to the adaptation of a limited

number of LAB species to the specific conditions prevailing during sourdough fermentation and/or stems from the persistence of certain LAB strain types.

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