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Influence of Geographical Origin and Flour Type on Diversity of Lactic Acid Bacteria in Traditional Belgian Sourdoughs▽†

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A culture-based approach was used to investigate the diversity of lactic acid bacteria (LAB) in Belgian traditional sourdoughs and to assess the influence of flour type, bakery environment, geographical origin, and technological characteristics on the taxonomic composition of these LAB communities. For this purpose, a total of 714 LAB from 21 sourdoughs sampled at 11 artisan bakeries throughout Belgium were subjected to a polyphasic identification approach. The microbial composition of the traditional sourdoughs was characterized by bacteriological culture in combination with genotypic identification methods, including repetitive element sequence-based PCR fingerprinting and phenylalanyl-tRNA synthase (*pheS*) gene sequence analysis. LAB from Belgian sourdoughs belonged to the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Weissella*, and *Enterococcus*, with the heterofermentative species *Lactobacillus paralimentarius*, *Lactobacillus sanfranciscensis*, *Lactobacillus plantarum*, and *Lactobacillus pontis* as the most frequently isolated taxa. Statistical analysis of the identification data indicated that the microbial composition of the sourdoughs is mainly affected by the bakery environment rather than the flour type (wheat, rye, spelt, or a mixture of these) used. In conclusion, the polyphasic approach, based on rapid genotypic screening and high-resolution, sequence-dependent identification, proved to be a powerful tool for studying the LAB diversity in traditional fermented foods such as sourdough.

Sourdough represents a natural food ecosystem in which the fermentation activities of lactic acid bacteria (LAB) and yeasts largely determine the typical characteristics of the resulting baked goods (26). Based on the production technology, sourdough fermentations can be divided into three types (2, 38): type I, or traditional, sourdoughs are characterized by continuous propagation of the dough at ambient temperatures (20 to 30°C); type II, or industrial, sourdoughs are incubated at high temperatures (>30°C), with longer fermentation times and a higher water content; and type III sourdoughs are dried preparations of industrial doughs. Traditional Belgian sourdoughs belong to type I sourdoughs. The use of sourdough improves the overall characteristics of bakery products, such as the dough properties, texture, and flavor (16). LAB contribute significantly to these properties, e.g., by acidification of the dough (17), proteolysis of the gluten (10), hydrolysis of the starch (5), and the production of taste and aromatic compounds (15). Moreover, several sourdough LAB inhibit the development of pathogens due to the production of acids and bacteriocins (22). During spontaneous sourdough maturation, LAB occur as the predominant microorganisms, at numbers of $>10^8$ CFU/g sourdough. The genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* predominate in this ecosystem, whereas lactococci, enterococci, and streptococci are rarely found. Sourdough LAB may originate from natural contamination of the flour or may be introduced into the ecosystem as part of a starter culture. Due to the large variety of cereals and fermentation conditions, the taxonomic composition of LAB microbiota found in sourdoughs worldwide is very diverse. In recent years, the number of new *Lactobacillus* species from sourdough origins has steadily increased (1, 3, 7, 11, 20, 23, 28, 29, 31, 32, 34, 36, 39).

The increasing interest in starter cultures for sourdough fermentations requires better insights into the genetic and phenotypic diversity of strains for exploitation in technological processes. Although still important for the description of new species, conventional methods such as carbohydrate fermentation pattern analysis and cell wall analyses are not reliable for the accurate identification of LAB at the species level. The use of 16S rRNA gene sequencing is generally regarded as a more reliable solution for the classification and identification of LAB, although the differentiation of closely related *Lactobacillus* species is not always straightforward, due to a high degree of conservation within this genus (12). Alternatively, sequence-based identification using one or more protein-coding genes has been explored as an alternative to determine the genomic relatedness between LAB strains (4). In this regard, the phenylalanyl-tRNA synthase gene (*pheS*) has proven to be a valuable tool for the identification of *Lactobacillus* species and the delineation of novel taxa (24, 24a, 28, 33).

The objective of the present study was to assess the taxonomic diversity of LAB isolated from traditional sourdoughs

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TABLE 1. Main characteristics of 21 Belgian traditional sourdoughs included in this study[a]

Sample designation	Province	Fermentation time (h)	Fermentation temp (°C)	pH	Log CFU/g (SD)		No. of LAB isolates recovered
					Yeasts	LAB	
D01WW01T01	East Flanders	10	30	3.83	7.68 (6.79)	8.9 (8.18)	40
D02WR01T01	West Flanders	<24	AT	4.01	4.2 (3.84)	8.86 (8.74)	31
D02WR01T02	West Flanders	22	AT	3.80	3.89 (3.40)	9.02 (8.66)	26
D02WW01T01	West Flanders	22	AT	3.83	5.72 (4.62)	9.15 (8.34)	29
D03WW01T01	West Flanders	4	30	4.12	7.57 (6.53)	8.97 (8.32)	40
D04WW01T01	Brabant Wallon	0	AT	5.00	3.12 (3.06)	8.29 (8.14)	38
D05WW01T01	East Flanders	24	28	3.63	7.14 (6.09)	8.6 (8.58)	40
D06SS01T01	Liège	2	21–23	5.04	4.55 (3.23)	7.67 (6.61)	40
D06WW01T01	Liège	2	21–23	4.75	4.77 (3.85)	7.5 (7.59)	41
D07WR01T01	Hainaut	13	26	3.83	7.46 (6.38)	8.67 (8.42)	41
D07WR01T02	Hainaut	13	26	3.86	7.46 (6.57)	8.19 (8.07)	40
D07WR02T01	Hainaut	12	AT	3.78	6.98 (5.99)	8.9 (7.44)	39
D08WW01T01	Namur	6	AT	3.80	6.84 (6.17)	7.99 (7.11)	40
D09ME01T01	Namur	<24	AT	3.78	7.54 (6.59)	8.68 (7.94)	41
D10WW01T01	Luxembourg	4	28	4.09	5.94 (4.06)	8.3 (8.56)	9
D10SS01T01	Luxembourg	4	28	4.28	5.63 (4.96)	8.03 (8.31)	15
D10WR01T01	Luxembourg	4	28	3.96	5.92 (5.15)	8.14 (8.43)	7
D11RR01T01	Namur	12	AT	3.79	7.67 (6.88)	9.25 (8.38)	38
D11WW01T01	Namur	12	AT	3.72	7.26 (6.50)	9.08 (8.11)	40
D11WW02T01	Namur	12	AT	3.85	7.62 (6.81)	9.17 (8.33)	40
D11SS01T01	Namur	12	AT	3.83	7.29 (6.69)	9.02 (8.19)	40

[a] D, depositor; WW, wheat flour; RR, rye flour; SS, spelled flour; WR, mixture of wheat and rye flour; ME, mixture of wheat, rye and spelt flour; T, time of sampling; AT, ambient temperature.

collected from local bakeries throughout different provinces of Belgium. The assessment of the LAB biodiversity was based entirely on genotypic methods, such as repetitive DNA element PCR (rep-PCR) fingerprinting and sequencing of the *pheS* gene. The rep-PCR method was used to reduce the initial collection of LAB isolates to a set of genotypically unique isolates and allowed the tentative identification of the majority of sourdough LAB isolates. These identifications were further confirmed by *pheS* sequence analysis, and the taxonomic position of one *pheS* cluster that could not be allocated to any of the LAB species included in the *pheS* reference database was characterized by 16S rRNA gene sequence analysis and DNA-DNA hybridization experiments. Using statistical analysis, the influence of geographical origin, flour type, bakery environment, and technological characteristics on the LAB biodiversity in Belgian sourdoughs was assessed.

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. (21). All LAB reference strains were obtained from the BCCM/LMG Bacteria Collection, Ghent University (http://bccm.belspo.be; Ghent, Belgium).

Sourdough sampling and isolation of LAB and yeasts. A total of 11 different bakeries from seven provinces throughout Belgium were selected for sampling (Table 1). Samples from 21 natural sourdoughs were collected aseptically and kept at 4°C. Microbiological analyses were performed within 24 h after sampling. For microbiological analyses, sourdough samples (10 g) were homogenized in 90 ml peptone physiological solution (0.1% [wt/vol] bacteriological peptone [L37; Oxoid, Basingstoke, Hampshire, United Kingdom] and 0.85% [wt/vol] NaCl) by using a Stomacher 80 lab blender (Seward Medical, London, United Kingdom), after which serial dilutions in peptone physiological solution were prepared. LAB and yeasts were isolated on MRS-5 agar containing 0.1 g of cycloheximide (C-7698; Sigma-Aldrich Co., St. Louis, MO) and yeast-glucose (YG) agar (21a) supplemented with 0.1 g of chloramphenicol (C-0378; Sigma-Aldrich Co.) per liter, respectively. Routinely, 50 μl of the 10^{-4} to 10^{-7} dilutions was plated in triplicate on a series of MRS-5 agar plates that were incubated at four different incubation conditions: aerobic at 30°C, aerobic at 37°C, anaerobic at 30°C, and anaerobic at 37°C. Additionally, on YG plates, 50 μl of the 10^{-1} to 10^{-6} dilutions was plated on YG agar and aerobically incubated at 30°C (21a). Following incubation, an average of 40 colonies per sourdough sample was randomly selected from MRS-5 agar plates containing between 20 and 200 colonies. The colonies were further purified and stored in Microbank vials at −80°C. From YG agar plates, 10 colonies were picked and stored at 4°C on YG slants overlaid with sterile tap water. After the isolation, the remainder of the sourdough samples were stored at −20°C. A uniform code was assigned to each sourdough sample, encoding information on the depositor (D01 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; and ME, mixture of wheat, rye, and spelt flour), and the time of sampling (T). The main technological characteristics of the 21 sourdough samples are shown in Table 1.

Physiological characterization of sourdough isolates. The Gram reaction type and catalase activity were determined for isolates from the MRS-5 medium and the cell morphology was determined. Presumptive LAB isolates (gram-positive and catalase-negative bacilli and cocci) were selected for further characterization and identification.

rep-PCR genomic fingerprinting of LAB isolates. For the establishment of a taxonomical reference framework, a total of 63 type and reference strains belonging to 36 LAB species associated with sourdough were subjected to $(GTG)_5$-PCR fingerprinting. The genomic DNA of sourdough LAB was extracted from single colonies grown on MRS-5 agar plates by alkaline lysis. For this purpose, a small portion of one colony was resuspended in 20 μl lysis buffer (2.5 ml 10% sodium dodecyl sulfate, 5.0 ml 1 N NaOH, and 92.5 ml Milli-Q water). The mixture was heated at 95°C for 15 min and cooled immediately on ice, and 180 μl sterile Milli-Q water was added after a short centrifugation (1 s) at 13,000 × *g*. Subsequently, the mixture was centrifuged at 13,000 × *g* for 5 min and stored at −20°C. If the alkaline lysis method did not produce high quality $(GTG)_5$-PCR profiles, a phenol-chloroform method was used (13) and DNA work solutions of 50 ng/μl were prepared. For rep-PCR, the oligonucleotide primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) was used under the PCR conditions previously described (35). PCR amplifications were performed in a Perkin-Elmer 9600 PCR apparatus as previously described (19). PCR products were separated in a 1.5% agarose gel (15 by 20 cm) for 16 h at a constant voltage of 55 V in 1× TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 4°C. $(GTG)_5$-PCR profiles were visualized under UV light after being stained with ethidium bromide, followed by digital image capturing using a charge-coupled-device camera. The resulting fingerprints were analyzed by using the BioNumerics version 4.61 software package (Applied Maths, Sint-Martens-Latem, Belgium). The similarities among the

digitized profiles were calculated by using the Pearson correlation (expressed for convenience as a percentage-of-similarity value) and a dendrogram was derived from the profiles by using the unweighted pair-group method using arithmetic averages.

Phenylalanyl-tRNA synthase (*pheS*) gene sequencing of selected LAB isolates. For *pheS* sequence analysis, genomic DNA was obtained by alkaline lysis (see above). If the alkaline lysis method did not produce good-quality *pheS* sequence data, a phenol-chloroform method was used (13). The amplification conditions and sequencing reactions, using PheS-21-F and PheS-23-R, were those described by Naser et al. (24). The taxonomic reference framework of *pheS* sequences consisted of sequences of 255 *Lactobacillus* strains (representing 94 species), 51 *Enterococcus* strains (representing 9 species), 3 *Lactococcus* strains (representing 2 species), 41 *Leuconostoc* strains (representing 12 species), 26 *Pediococcus* strains (representing 9 species), and 20 *Weissella* strains (representing 10 species). Each of these species was represented by its type strain and, generally, by two to five additional reference strains. Newly obtained *pheS* sequences were imported into the BioNumerics version 4.61 software (Applied Maths), aligned, and compared using the neighbor-joining method with sequences available from the *pheS* database.

16S rRNA gene sequencing. Genomic DNA was extracted using the alkaline lysis method (see above) or a phenol-chloroform method (13). 16S rRNA gene amplification, purification, and sequencing were performed as described by Vancanneyt et al. (33). Phylogenetic analysis was performed using the software package BioNumerics version 4.61 (Applied Maths). To determine the closest relatives of 16S rRNA gene sequences, a search was performed in GenBank using the BLAST algorithm.

DNA-DNA hybridization experiments. For the extraction of high-molecular-mass DNA, strains were cultivated in MRS-5 broth. DNA-DNA hybridizations were performed as described previously (28), at a temperature of 37°C in the presence of 50% formamide.

Data analysis. All data processing and statistical analysis were performed using the software package BioNumerics version 4.61 (Applied Maths). The numerical data input was handled as an open character type, meaning that a nonfixed number of characters (i.e., the bacterial species) were allowed. For each sourdough sample, a microbial community profile was composed that reflects the qualitative (number of species) and quantitative (number of isolates per species) diversity of sourdough LAB in the corresponding sample. Similarities were expressed using the Pearson product-moment correlation coefficient, and a dendrogram was constructed using the unweighted pair-group method using arithmetic averages. In addition, principal component analysis (PCA) was carried out to visualize the possible impact of several technological characteristics on the sourdough LAB biodiversity.

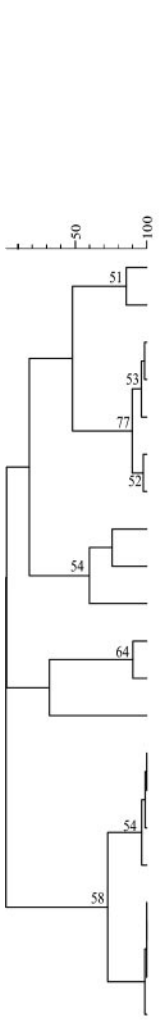
RESULTS

Enumeration of LAB and yeasts. The results of microbiological analysis (Table 1) showed that LAB constituted the major microbiota of the sourdough samples. LAB counts on MRS-5 agar ranged from 10^7 to 10^9 CFU/g sourdough, whereas yeast counts on YG agar ranged from 10^3 to 10^7 CFU/g sourdough. In general, the yeast-LAB ratio varied from about 1:10 to 1:100 (data not shown), except for samples D06SS01T01 and D07WR01T02, where the ratio was approximately 1:1, and samples D02WR01T01, D02WR01T02, and D04WW01T01, where the ratio varied from 1:10,000 to 1:100,000. The LAB counts on MRS-5 medium were comparable under the four incubation conditions, and no apparent differences in colonial morphology were noticed. The latter finding is also reflected in the LAB diversity of the sourdough samples, which was similar under the four conditions (data not shown). The mean bacterial colony counts in the sourdough samples are shown in Table 1.

Polyphasic identification of sourdough LAB isolates. $(GTG)_5$-PCR fingerprinting was used to screen 714 presumptive LAB isolates. Numerical analysis of the digitized $(GTG)_5$-PCR fingerprints with the reference database resulted in the delineation of 24 clusters and six single isolates at 50% Pearson similarity. Visual inspection of $(GTG)_5$-PCR fingerprints indicated that the variations in the band patterns within one rep-PCR cluster were mainly due to differences in band intensities. However, minor qualitative differences revealed heterogeneity among isolates from sourdoughs produced at different bakeries, suggesting that such isolates represent different strains. Isolates were tentatively assigned to a given species when they belonged to a $(GTG)_5$-PCR cluster that contained the type strain and/or one or more additional reference strains. This allowed the tentative identification of 514 LAB isolates (72% of the total) belonging to 10 different $(GTG)_5$-PCR clusters. The reproducibility of each run was evaluated by the inclusion of control strain *Lactobacillus alimentarius* LMG 21683. No marked qualitative variations between runs were observed for this strain (data not shown).

Subsequently, one or more representative strains from each $(GTG)_5$-PCR cluster were selected and subjected to *pheS* sequence analysis (a total of 96 isolates). The GenBank/EMBL/DDBJ accession numbers for the *pheS* gene sequences of the selected isolates are AM745639 to AM745731, AM285025, AM285026, and AM259120. For the identification of *Lactobacillus* and *Enterococcus* species, the *pheS* gene generally offers an intraspecies interval with a variation of up to 3% for both genera and interspecies gaps exceeding 10% and 16% divergence, respectively (90% confidence interval) (24; Naser et al., unpublished results). Data on the *pheS* intra- and interspecies variations for *Pediococcus*, *Leuconostoc*, and *Weissella* species are currently not available. Alignment and cluster analysis of the *pheS* sequences of the selected LAB sourdough isolates and reference strains resulted in the delineation of 25 *pheS* clusters at a similarity level of 97% (see Fig. S1 in the supplemental material). In this way, 91 LAB sourdough isolates (95%) belonging to 24 different *pheS* clusters could be identified at the species level. One *pheS* cluster that showed the highest *pheS* sequence similarity (94.2%) with the type strain of *Lactobacillus rossiae* (LMG 22972^T) could not be unequivocally allocated to a LAB species included in the reference database. Based on 16S rRNA gene sequencing, isolate D03WW01T01-35 of this "unknown" *pheS* cluster showed 99.5% 16S rRNA gene sequence similarity with *L. rossiae* LMG 22972^T. A DNA-DNA binding value of 83% was found between the type strain of *L. rossiae* (LMG 22972^T) and strain D03WW01T01-35, which is well above the proposed threshold of 70% for species delineation (30). It thus indicates that strain D03WW01T01-35 and all other members of the unidentified *pheS* cluster belong to *L. rossiae*.

Taxonomic distribution and prevalence of sourdough LAB species. Seven hundred and fourteen LAB were randomly isolated from 21 Belgian traditional sourdoughs. By polyphasic identification, 82% of the isolates were assigned to seven species: *Lactobacillus paralimentarius* (21%), *Lactobacillus sanfranciscensis* (17%), *Lactobacillus plantarum* (11%), *Lactobacillus pontis* (10%), *Lactobacillus fermentum* (9%), *Lactobacillus hammesii* (8%), and *Weissella cibaria* (6%). The heterofermentative species *L. paralimentarius*, *L. sanfranciscensis*, *L. plantarum*, and *L. pontis* occurred in 10, 9, 9, and 9 sourdoughs, respectively, and are therefore considered the dominant cultured microbiota in Belgian sourdoughs. However, all four species were never found together in any of the sourdoughs analyzed, and only in samples D01WW01T01 and D06WW01T01 was an



L. paralimentarius	*L. sanfranciscensis*	*L. plantarum*	*L. pontis*	*L. fermentum*	*L. hammesii*	*W. cibaria*	Sample code	Depositor	Type of flour
9		5	9				D01WW01T01	1	Wheat
22			12				D03WW01T01	3	Wheat
19					14		D09ME01T01	9	Wheat - Rye -Spelt
20		4			14		D11WW02T01	11	Wheat
26		1			11		D11RR01T01	11	Rye
15		7			12		D11SS01T01	11	Spelt
13		8			8		D11WW01T01	11	Wheat
9			15	14			D07WR01T01	7	Wheat - Rye
16			5	15			D07WR01T02	7	Wheat - Rye
			3	33			D07WR02T01	7	Wheat - Rye
		10				26	D06SS01T01	6	Spelt
2	7	12				17	D06WW01T01	6	Wheat
		27					D08WW01T01	8	Wheat
	15		14				D05WW01T01	5	Wheat
	4		4				D10WW01T01	10	Wheat
	4		3				D10WR01T01	10	Wheat - Rye
	9		5				D10SS01T01	10	Spelt
	26						D02WR01T02	2	Wheat - Rye
	28						D02WW01T01	2	Wheat
	12						D04WW01T01	4	Wheat
	18	3					D02WR01T01	2	Wheat - Rye

FIG. 1. Biodiversity data were imported into BioNumerics version 4.61 as an open character type, resulting in microbial community profiles reflecting the diversity of dominant LAB species in 21 Belgian traditional sourdoughs analyzed. Descriptive (depositor and type of flour) characteristics are shown; technological characteristics (pH and fermentation time and temperature) are in Table 1. The dendrogram was constructed by using the unweighted-pair group method using arithmetic averages, with correlation levels expressed as percentage-of-similarity values of the Pearson correlation coefficient. Bootstrap values (based on 1,000 replications) at or above 50% are indicated at the branch points.

association of three of the species observed. Furthermore, the occurrence of the sourdough species *L. sanfranciscensis* and *L. paralimentarius* seemed to be negatively correlated (Spearman correlation value, $\rho = -0.656$; $P = 0.000625$). *L. fermentum*, *L. hammesii*, and *W. cibaria* occurred in three, five, and two sourdoughs, respectively.

The remaining 129 isolates (18%) were assigned to LAB species *Lactobacillus sakei* ($n = 26$), *Lactobacillus brevis* ($n = 15$), *Lactobacillus helveticus* ($n = 15$), *L. rossiae* ($n = 11$), *Lactobacillus curvatus* ($n = 11$), *Pediococcus pentosaceus* ($n = 9$), *Lactobacillus parabuchneri* ($n = 8$), *Lactobacillus spicheri* ($n = 8$), *Lactobacillus nantensis* ($n = 7$), *Lactobacillus namurensis* ($n = 6$), *Lactobacillus crustorum* ($n = 3$), *Pediococcus acidilactici* ($n = 3$), *Leuconostoc mesenteroides* ($n = 2$), *Weissella confusa* ($n = 2$), *Enterococcus mundtii* ($n = 1$), *Lactobacillus buchneri* ($n = 1$), and *Lactobacillus paracasei* ($n = 1$). The microbiota of the sampled sourdoughs consisted of two to seven different LAB species, with the exception of D02WR01T02, from which only *L. sanfranciscensis* was isolated. The recently described *Lactobacillus* species *L. namurensis* (28) and *L. crustorum* (29) were isolated from one (D09ME01T01) and two (D01WW01T01 and D05WW01T01) sourdoughs, respectively. Notably, the occurrence of *L. crustorum* and *L. helveticus* seemed to be positively correlated in the sampled sourdoughs (Spearman correlation value, $\rho = 0.995$; $P < 0.01$).

To study the influence of descriptive and technological characteristics on the LAB diversity and the relationships between dominant LAB species in the sourdough samples, cluster and PCA analyses were performed. Seven bacterial species, corresponding to the seven most-dominant species that together represent 82% of the total LAB population, were included in the analyses (Fig. 1). Except for sample D07WR02T01 from depositor 7, all sourdough samples produced at one particular bakery were found together in separate groups and tended to show a similar qualitative and quantitative microbial diversity.

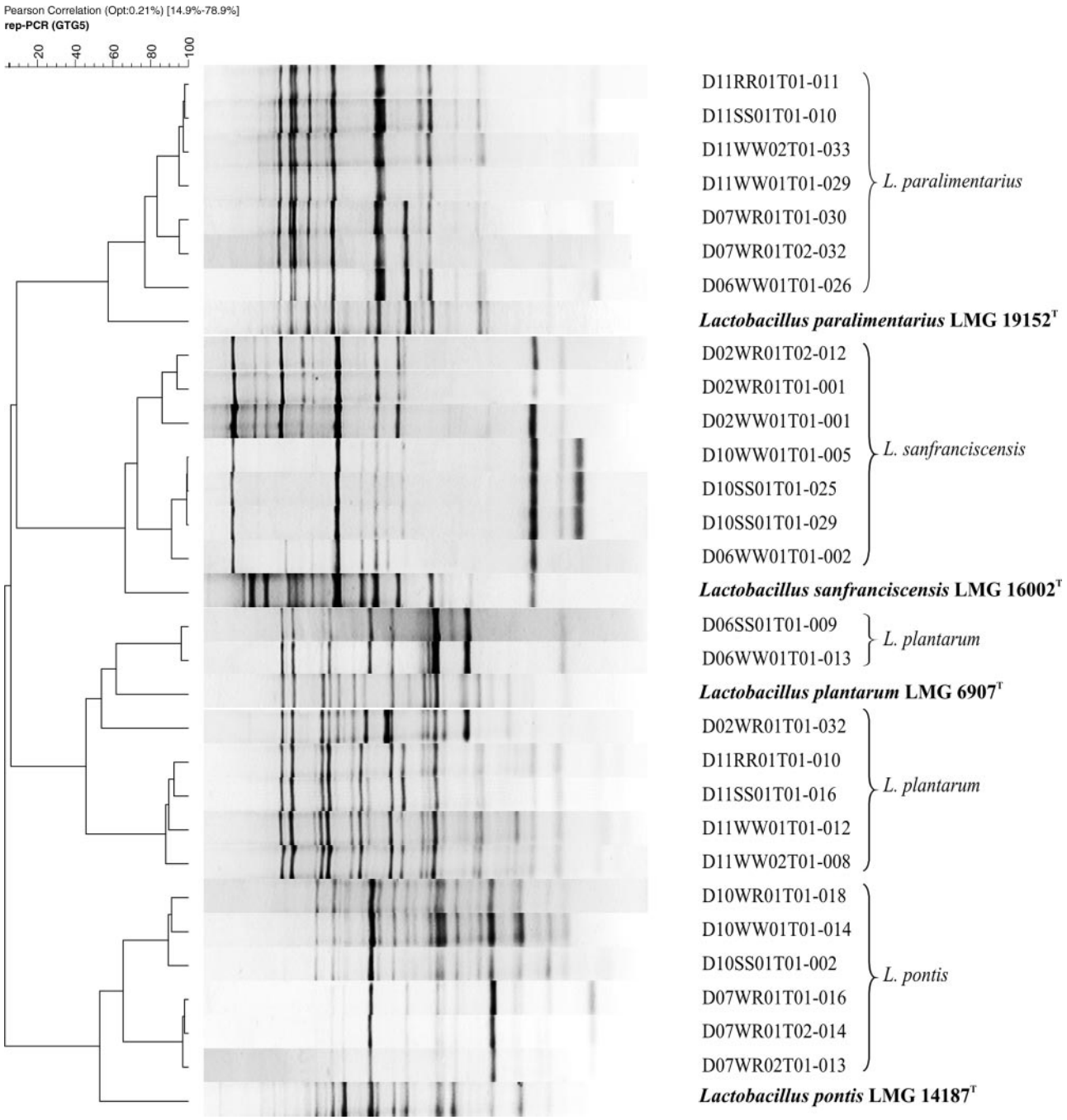


FIG. 2. Dendrogram showing the genetic similarities between selected strains of the four most frequently isolated species from Belgian artisan sourdoughs (*L. paralimentarius*, *L. sanfranciscenis*, *L. plantarum*, and *L. pontis*) based on $(GTG)_5$-PCR fingerprints. One representative fingerprint pattern per species isolated from sourdough samples originating from depositors D2, D6, D7, D10, and D11 and the pattern of the corresponding type strain (in bold face) were included for cluster analysis. The dendrogram was constructed using the unweighted-pair group method using arithmetic averages, with correlation levels expressed as percentage-of-similarity values of the Pearson correlation coefficient.

As an example, all sourdough samples originating from depositor 11 and produced with different types of flour (rye, spelt, and two varieties of wheat flour) clearly grouped together and are all dominated by the species *L. paralimentarius*, *L. plantarum*, and *L. hammesii* (Fig. 1). On the other hand, a number of sourdoughs from different bakeries (e.g., those from depositor 5 and 10) displayed highly similar LAB compositions.

In Fig. 2, the interstrain variation based on $(GTG)_5$-PCR cluster analysis among isolates of the species most frequently isolated from Belgian sourdoughs, i.e., *L. paralimentarius*, *L. sanfranciscensis*, *L. plantarum*, and *L. pontis*, is shown. Generally, no pronounced qualitative differences were observed among the band patterns of isolates of the same species originating from the same depositor. On the other hand,

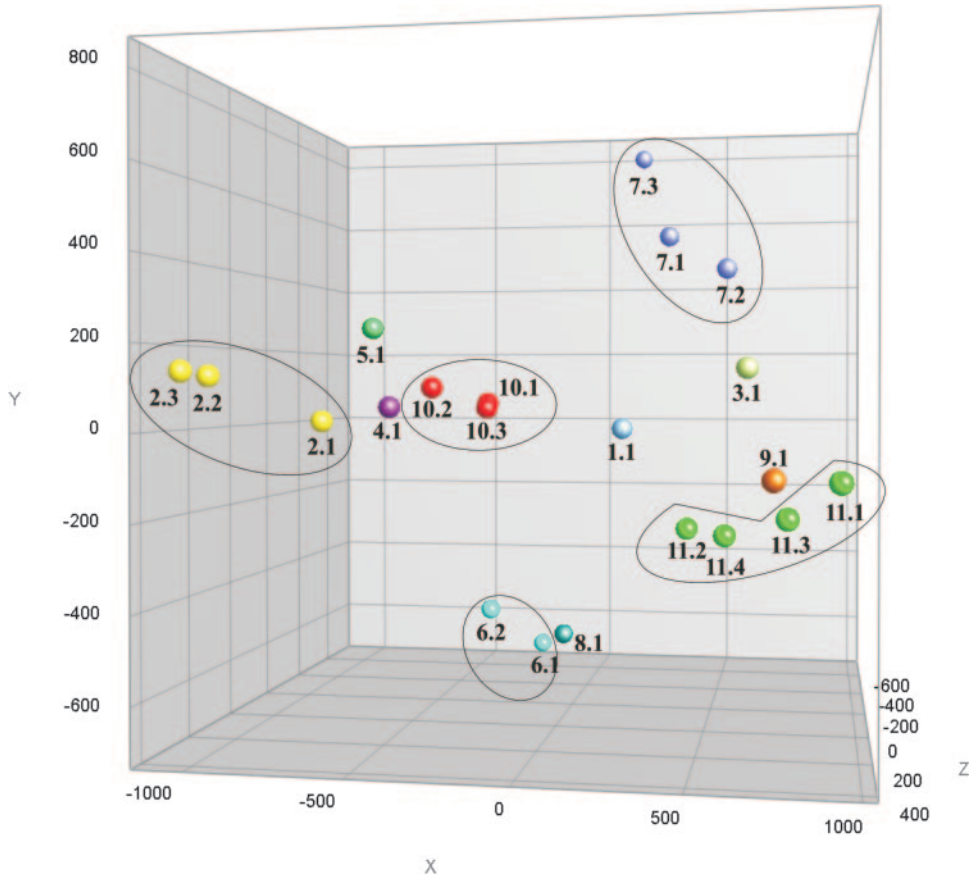


FIG. 3. Three-dimensional score plot of the PCA of the data obtained from 21 Belgian artisan sourdough samples. 1.1, D01WW01T01; 2.1, D02WR01T01; 2.2, D02WR01T02; 2.3, D02WW01T01, 3.1, D03WW01T01; 4.1, D04WW01T01; 5.1, D05WW01T01; 6.1, D01SS01T01; 6.2, D01WW01T01; 7.1, D07WR01T01; 7.2, D07WR01T02; 7.3, D07WR02T01; 8.1, D08WW01T01; 9.1, D09ME01T01; 10.1, D10WW01T01; 10.2, D10SS01T01; 10.3, D10WR01T01; 11.1; D11RR01T01, 11.2, D11WW01T01; 11.3, D11WW02T01; 11.4, D11SS01T01.

minor infraspecific band pattern variations could be detected among isolates purified from sourdoughs produced at different bakeries.

The score plot of a first PCA revealed that sourdough samples originating from the same depositor clustered together (Fig. 3). Still, other sourdoughs (e.g., D09ME01T01) clustered together with sourdoughs produced at another bakery (e.g., D11RR01T01, D11SS01T01, D11WW01T01, and D11WW02T01). In addition, no obvious correlation between the type of flour, province, or technological characteristics (pH, fermentation time, and temperature) and the microbial composition of the analyzed sourdoughs and no significant associations of certain LAB species with one of these variable factors could be observed. A second PCA was performed on a subset of the data from samples originating from depositors producing more than one sourdough (i.e., D2, D6, D7, D10, and D11). The resulting score plot can be divided into five major groups corresponding to the five depositors (see Fig. S2 in the supplemental material). Furthermore, both PCA analyses indicated that certain species were positively correlated with specific depositors, i.e., *L. sanfranciscensis* with depositor 2 and *L. fermentum* with depositor 7.

DISCUSSION

In this study, 714 LAB isolates originating from 21 Belgian traditional sourdoughs were characterized by using a polyphasic strategy for the screening of a large number of isolates and subsequent identification of genotypically unique strains. Comparison of $(GTG)_5$-PCR fingerprints with an in-house reference database allowed the identification of 72% of the isolates. Members of the species *L. sanfranciscensis*, *L. plantarum*, *L. curvatus*, *L. rossiae*, and *W. confusa* were allocated to more than one $(GTG)_5$-PCR cluster (data not shown). Visual inspection of the fingerprint patterns demonstrated that the observed partition in the cluster analysis of the *W. confusa*, *L. plantarum*, and *L. curvatus* isolates was mainly due to variations in band intensities. In contrast, the $(GTG)_5$-PCR patterns of *L. sanfranciscensis* strains varied in the number and position of the bands. As only a few bands (two to four) were observed in the $(GTG)_5$-PCR profiles of these strains, minor qualitative shifts in the band pattern composition drastically affected their relative grouping and resulted in the formation of multiple $(GTG)_5$-PCR clusters. The finding of two clusters for *L. rossiae* was further investigated with *pheS* sequencing. Based on the $(GTG)_5$-PCR clustering, representatives of all

clusters were selected for *pheS* sequence analysis and compared with an in-house reference database, which resulted in the identification of 95% of the selected isolates. The single "unknown" *pheS* cluster was assigned to *L. rossiae* following 16S rRNA gene sequence analysis and DNA-DNA hybridizations. This is the first study to demonstrate the usefulness of *pheS* gene sequence analysis for the identification of a large collection of unknown LAB isolates. Except for *L. rossiae*, this method proved to be very valuable for the discrimination of sourdough LAB species. It is beyond doubt that the construction of a *pheS* sequence database that could be freely accessed online would be a promising development for the fast and reproducible identification of LAB species involved in food fermentations. Thus, the combined use of $(GTG)_5$-PCR fingerprinting and *pheS* sequence analysis proved to be very efficient in the differentiation and identification of a large set of sourdough LAB isolates in a relatively economical and fast manner.

Consistent with the results of other European sourdough biodiversity studies, heterofermentative species appear to dominate the microbiota of Belgian type I sourdoughs (6, 9, 14, 36), although complex associations of homo- and heterofermentative strains were found in 12 of the 21 sourdoughs. All Belgian sourdough samples were dominated by one or more of the heterofermentative species *L. paralimentarius*, *L. sanfranciscensis*, *L. plantarum*, and *L. pontis*. The relative dominance of *L. sanfranciscensis* in type I sourdoughs has been ascribed to environmental conditions during sourdough fermentation (6, 8, 27, 37). In contrast to these studies, in which the frequent association of *L. sanfranciscensis* and *L. plantarum* is reported, we detected both species in only 2 of the 21 Belgian sourdoughs. Likewise, Corsetti et al. (6) suggested an association of *L. sanfranciscensis* and *L. alimentarius* in Italian wheat sourdoughs, but no members of the latter species were found in the present study. However, the closely related *L. paralimentarius* (3) is common to traditional Greek wheat sourdoughs (9) and was isolated from 10 Belgian sourdoughs produced at six different bakeries. In addition, our results suggest that the copresence of *L. sanfranciscensis* and *L. paralimentarius* in Belgian sourdough samples is negatively correlated. Interestingly, the negative effect of *L. paralimentarius* on *L. sanfranciscensis* growth has been previously reported (25). Another characteristic of Belgian sourdoughs is the presence of the obligate heterofermentative species *L. pontis*, which is frequently isolated from European sourdoughs (37, 38) and was found in 9 of the 21 Belgian sourdoughs sampled. However, *L. pontis* has been isolated from pig intestines as well, indicating that *L. pontis* is not associated only with sourdough (18). The rare presence of the recently described LAB species *L. namurensis* (28) and *L. crustorum* (29) in one and two Belgium sourdoughs, respectively, may be the result of random colony picking or nonoptimal culture conditions (8). Hence, culture-independent studies using denaturing gradient gel electrophoresis and/or real-time PCR are expected to yield more information about the relative abundance and ecology of these and other species in Belgian sourdoughs.

Although it is known that the type of flour, process technology, and other factors strongly influence the composition of the sourdough microbiota (9, 21), cluster analysis and PCA indicate that the typical microbial composition of Belgian sourdoughs is influenced by the bakery environment, rather than the type of flour used to produce the sourdough. Individual exceptions to this conclusion may be the result of arbitrary colony selection. Although it was beyond the scope of this study to investigate the clonal relatedness of the sourdough isolates in depth, $(GTG)_5$-PCR fingerprint analysis gave evidence of high infraspecific similarity among isolates from sourdoughs produced at the same bakery. These results may indicate that the "in-house microbiota" of the bakery setting largely determines the microbial diversity in sourdoughs.

In conclusion, the combined use of $(GTG)_5$-PCR fingerprinting and *pheS* sequence analysis proved to be a valuable method for the screening and identification of a large set of LAB sourdough isolates. This polyphasic approach was applied for the analysis of 21 Belgian sourdough samples originating from 11 different artisan bakeries located throughout Belgium. Large differences in species composition were found, except in sourdoughs from the same bakery, which demonstrated a similar qualitative and quantitative LAB population. These findings suggest that the bakery environment significantly influences the LAB community structure of Belgian sourdoughs, irrespective of the type of flour used to prepare the sourdough.

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REFERENCES

1. **Aslam, Z., W. T. Im, L. N. Ten, M. J. Lee, K. H. Kim, and S. T. Lee.** 2006. *Lactobacillus siliginis* sp. nov., isolated from wheat sourdough in South Korea. Int. J. Syst. Evol. Microbiol. **56:**2209–2213.
2. **Böcker, G., P. Stolz, and W. P. Hammes.** 1995. Nueu Erkentnisse zum Ökosystel Sauerteig und zur Physiologie der sauerteig-typischen Stämme *Lactobacillus sanfranciscensis* und *Lactobacillus pontis*. Getreide Mehl und Brot **49:**370–374.
3. **Cai, Y. M., H. Okada, H. Mori, Y. Benno, and T. Nakase.** 1999. *Lactobacillus paralimentarius* sp. nov., isolated from sourdough. Int. J. Syst. Bacteriol. **49:**1451–1455.
4. **Cooper, J. E., and E. J. Feil.** 2004. Multilocus sequence typing: what is resolved? Trends Microbiol. **12:**373–377.
5. **Corsetti, A., M. Gobbetti, F. Balestrieri, F. Paoletti, L. Russi, and J. Rossi.** 1998. Sourdough lactic acid bacteria effects on bread firmness and staling. J. Food Sci. **63:**347–351.
6. **Corsetti, A., P. Lavermicocca, M. Morea, F. Baruzzi, N. Tosti, and M. Gobbetti.** 2001. Phenotypic and molecular identification and clustering of lactic acid bacteria and yeasts from wheat (species *Triticum durum* and *Triticum aestivum*) sourdoughs of Southern Italy. Int. J. Food Microbiol. **64:**95–104.
7. **Corsetti, A., L. Settanni, D. van Sinderen, G. E. Felis, F. Dellaglio, and M. Gobbetti.** 2005. *Lactobacillus rossii* sp. nov., isolated from wheat sourdough. Int. J. Syst. Evol. Microbiol. **55:**35–40.
8. **De Vuyst, L., and P. Neysens.** 2005. The sourdough microflora: biodiversity and metabolic interactions. Trends Food Sci. Technol. **16:**43–56.
9. **De Vuyst, L., V. Schrijvers, S. Paramithiotis, B. Hoste, M. Vancanneyt, J. Swings, G. Kalantzopoulos, E. Tsakalidou, and W. Messens.** 2002. The biodiversity of lactic acid bacteria in Greek traditional wheat sourdoughs is reflected in both composition and metabolite formation. Appl. Environ. Microbiol. **68:**6059–6069.
10. **Di Cagno, R., M. De Angelis, P. Lavermicocca, M. De Vincenzi, C. Giovannini,**

M. Faccia, and M. Gobbetti. 2002. Proteolysis by sourdough lactic acid bacteria: effects on wheat flour protein fractions and gliadin peptides involved in human cereal intolerance. Appl. Environ. Microbiol. **68:**623–633.
11. **Ehrmann, M. A., M. R. A. Müller, and R. F. Vogel.** 2003. Molecular analysis of sourdough reveals *Lactobacillus mindensis* sp. nov. Int. J. Syst. Evol. Microbiol. **53:**7–13.
12. **Ehrmann, M. A., and R. F. Vogel.** 2005. Molecular taxonomy and genetics of sourdough lactic acid bacteria. Trends Food Sci. Technol. **16:**31–42.
13. **Gevers, D., G. Huys, and J. Swings.** 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiol. Lett. **205:**31–36.
14. **Gobbetti, M., A. Corsetti, J. Rossi, F. La Rosa, and S. Vincenzi.** 1994. Identification and clustering of lactic acid bacteria and yeasts from wheat sourdoughs of central Italy. Ital. J. Food Sci. **1:**85–94.
15. **Gobbetti, M., M. S. Simonetti, A. Corsetti, F. Santinelli, J. Rossi, and P. Damiani.** 1995. Volatile compound and organic acid productions by mixed wheat sour dough starters: influence of fermentation parameters and dynamics during baking. Food Microbiol. **12:**497–507.
16. **Hammes, W. P., and M. G. Gänzle.** 1998. Sourdough breads and related products, p. 199–216. *In* B. J. B. Wood (ed.), Microbiology of fermented foods, vol. 1. Blackie Academic & Professional, London, United Kingdom.
17. **Leroy, F., and L. De Vuyst.** 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol. **15:**67–78.
18. **Leser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Moller.** 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl. Environ. Microbiol. **68:**673–690.
19. **Masco, L., G. Huys, D. Gevers, L. Verbrugghen, and J. Swings.** 2003. Identification of *Bifidobacterium* species using rep-PCR fingerprinting. Syst. Appl. Microbiol. **26:**557–563.
20. **Meroth, C. B., W. P. Hammes, and C. Hertel.** 2004. Characterisation of the microbiota of rice sourdoughs and description of *Lactobacillus spicheri* sp. nov. Syst. Appl. Microbiol. **27:**151–159.
21. **Meroth, C. B., J. Walter, C. Hertel, M. J. Brandt, and W. P. Hammes.** 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **69:**475–482.
21a. **Meroth, C. B., W. P. Hammes, and C. Hertel.** 2003. Identification and population dynamics of yeasts in sourdough fermentation processes by PCR-denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **69:**7453–7461.
22. **Messens, W., and L. De Vuyst.** 2002. Inhibitory substances produced by lactobacilli isolated from sourdoughs—a review. Int. J. Food Microbiol. **72:**31–43.
23. **Müller, M. R. A., M. A. Ehrmann, and R. F. Vogel.** 2000. *Lactobacillus frumenti* sp. nov., a new lactic acid bacterium isolated from rye-bran fermentations with a long fermentation period. Int. J. Syst. Evol. Microbiol. **50:** 2127–2133.
24. **Naser, S. M., F. L. Thompson, B. Hoste, D. Gevers, P. Dawyndt, M. Vancanneyt, and J. Swings.** 2005. Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. Microbiology **151:**2141–2150.
24a. **Naser, S. M., P. S. R. Dawyndt, B. Hoste, D. Gevers, K. Vandemeulebroecke, I. Cleenwerck, M. Vancanneyt, and J. Swings.** Identification of lactobacilli by *pheS* and *rpoA* gene sequence analyses. Int. J. Syst. Evol. Microbiol., in press.
25. **Paramithiotis, S., Y. Chouliaras, E. Tsakalidou, and G. Kalantzopoulos.** 2005. Application of selected starter cultures for the production of wheat sourdough bread using a traditional three-stage procedure. Process Biochem. **40:**2813–2819.
26. **Pepe, O., G. Blaiotta, M. Anastasio, G. Moschetti, D. Ercolini, and F. Villani.** 2004. Technological and molecular diversity of *Lactobacillus plantarum* strains isolated from naturally fermented sourdoughs. Syst. Appl. Microbiol. **27:**443–453.
27. **Randazzo, C. L., H. Heilig, C. Restuccia, P. Giudici, and C. Caggia.** 2005. Bacterial population in traditional sourdough evaluated by molecular methods. J. Appl. Microbiol. **99:**251–258.
28. **Scheirlinck, I., R. Van der Meulen, A. Van Schoor, I. Cleenwerck, G. Huys, P. Vandamme, L. De Vuyst, and M. Vancanneyt.** 2007. *Lactobacillus namurensis* sp. nov., isolated from a traditional Belgian sourdough. Int. J. Syst. Evol. Microbiol. **57:**223–227.
29. **Scheirlinck, I., R. Van der Meulen, A. Van Schoor, G. Huys, P. Vandamme, L. De Vuyst, and M. Vancanneyt.** *Lactobacillus crustorum* sp. nov., isolated from three traditional Belgian wheat sourdoughs. Int. J. Syst. Evol. Microbiol., in press.
30. **Stackebrandt, E., and B. M. Goebel.** 1994. A place for DNA-DNA reassociation and 16S ribosomal RNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
31. **Valcheva, R., M. F. Ferchichi, M. Korakli, I. Ivanova, M. G. Gänzle, R. F. Vogel, H. Prevost, B. Onno, and X. Dousset.** 2006. *Lactobacillus nantensis* sp. nov., isolated from French wheat sourdough. Int. J. Syst. Evol. Microbiol. **56:**587–591.
32. **Valcheva, R., M. Korakli, B. Onno, H. Prevost, I. Ivanova, M. A. Ehrmann, X. Dousset, M. G. Gänzle, and R. F. Vogel.** 2005. *Lactobacillus hammesii* sp. nov., isolated from French sourdough. Int. J. Syst. Evol. Microbiol. **55:**763–767.
33. **Vancanneyt, M., S. M. Naser, K. Engelbeen, M. De Wachter, R. Van der Meulen, I. Cleenwerck, B. Hoste, L. De Vuyst, and J. Swings.** 2006. Reclassification of *Lactobacillus brevis* strains LMG 11494 and LMG 11984 as *Lactobacillus parabrevis* sp. nov. Int. J. Syst. Evol. Microbiol. **56:**1553–1557.
34. **Vancanneyt, M., P. Neysens, M. De Wachter, K. Engelbeen, C. Snauwaert, I. Cleenwerck, R. Van der Meulen, B. Hoste, E. Tsakalidou, L. De Vuyst, and J. Swings.** 2005. *Lactobacillus acidifarinae* sp. nov. and *Lactobacillus zymae* sp. nov., from wheat sourdoughs. Int. J. Syst. Evol. Microbiol. **55:**615–620.
35. **Versalovic, J., M. Schneider, F. J. De Bruijn, and J. R. Lupski.** 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell. Biol. **5:**25–40.
36. **Vogel, R. F., G. Bocker, P. Stolz, M. Ehrmann, D. Fanta, W. Ludwig, B. Pot, K. Kersters, K. H. Schleifer, and W. P. Hammes.** 1994. Identification of lactobacilli from sourdough and description of *Lactobacillus pontis* sp. nov. Int. J. Syst. Bacteriol. **44:**223–229.
37. **Vogel, R. F., R. Knorr, M. R. A. Müller, U. Steudel, M. G. Gänzle, and M. A. Ehrmann.** 1999. Non-dairy lactic fermentations: the cereal world. Antonie Leeuwenhoek **76:**403–411.
38. **Vogel, R. F., M. R. A. Müller, P. Stolz, and M. A. Ehrmann.** 1996. Ecology in sourdough produced by traditional and modern technologies. Adv. Food Sci. **18:**152–159.
39. **Wiese, B. G., W. Strohmar, F. A. Rainey, and H. Diekmann.** 1996. *Lactobacillus panis* sp. nov., from sourdough with a long fermentation period. Int. J. Syst. Bacteriol. **46:**449–453.