Lactobacillus crustorum sp. nov., isolated from two traditional Belgian wheat sourdoughs

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A polyphasic taxonomic study of the lactic acid bacteria (LAB) population in three traditional Belgian sourdoughs, sampled between 2002 and 2004, revealed a group of isolates that could not be assigned to any recognized LAB species. Initially, sourdough isolates were screened by means of (GTG)₅-PCR fingerprinting. Four isolates displaying unique (GTG)₅-PCR patterns were further investigated by means of phenylalanyl-tRNA synthase (*pheS*) gene sequence analysis and represented a bifurcated branch that could not be allocated to any LAB species present in the in-house *pheS* database. Their phylogenetic affiliation was determined using 16S rRNA gene sequence analysis and showed that the four sourdough isolates belong to the *Lactobacillus plantarum* group with *Lactobacillus mindensis*, *Lactobacillus farciminis* and *Lactobacillus nantensis* as closest relatives. Further genotypic and phenotypic studies, including whole-cell protein analysis (SDS-PAGE), amplified fragment length polymorphism (AFLP) fingerprinting, DNA–DNA hybridization, DNA G + C content analysis, growth characteristics and biochemical features, demonstrated that the new sourdough isolates represent a novel *Lactobacillus* species for which the name *Lactobacillus crustorum* sp. nov. is proposed. The type strain of the new species is LMG 23699^T (=CCUG 53174^T).

Sourdough consists of a natural microbial consortium in which the fermentation activities of lactic acid bacteria (LAB) and yeasts determine the typical characteristics of baked goods (Hammes & Gänzle, 1998). Both groups of micro-organisms, present in the flour or added as a starter, contribute to the sourdough fermentation. While yeasts are primarily responsible for the leavening process, LAB mainly contribute to lactic fermentation, proteolysis, synthesis of aromatic compounds, and anti-mould and anti-ropiness activity (Gobbetti, 1998; Hammes & Gänzle, 1998). *Lactobacillus* species dominate in sourdough due to their competitiveness and adaptation to the specific environmental

conditions (De Vuyst & Neysens, 2005). The majority of recently described species isolated from sourdoughs are members of the *Lactobacillus plantarum* group and *Lactobacillus brevis* group, such as *Lactobacillus nantensis* (Valcheva *et al.*, 2006) and *Lactobacillus namurensis* (Scheirlinck *et al.*, 2007), respectively. The phylogenetic relatedness of species within these groups is strongly based on 16S rRNA gene sequence analysis (Rossello-Mora & Amann, 2001); however, in other bacterial taxa, the high degree of conservation of the 16S rRNA gene complicates the differentiation of such closely related species (Cooper & Feil, 2004). Recent work has indicated that the protein-encoding gene phenylalanyl-tRNA synthase (*pheS*) is a promising tool for delineation and identification of *Lactobacillus* species (S. M. Naser, P. S. R. Dawyndt, B. Hoste, D. Gevers, K. Vandemeulebroecke, I. Cleenwerck, M. Vancanneyt & J. Swings, unpublished; Vancanneyt *et al.*, 2006).

In the period 2002–2004, we obtained a group of *Lactobacillus* isolates from traditional Belgian wheat sourdoughs that could not be assigned to any hitherto known species. In

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 23699^T, LMG 23701, LMG 23702 and R-30103 are AM285450, AM285454, AM285451 and AM285452, respectively, and for the *pheS* gene sequences AM285025, AM285027, AM285028 and AM285449, respectively.

A figure showing data from numerical analysis of the protein profiles is available with the online version of this paper.

the present study, phenotypic and genotypic evidence is presented to support the description of this group as a novel *Lactobacillus* species for which the name *Lactobacillus crustorum* sp. nov. is proposed.

The wheat sourdough samples used in this study originate from biodiversity studies performed at two different Belgian artisan bakeries. Both wheat sourdoughs were incubated at 28–30 °C and used for the production of cookies, baguettes, hard rolls and bread. Sourdough samples were taken aseptically, stored at 4 °C and analysed within 24 h. Sourdough samples were suspended (1 : 10, w/v) and serially diluted in peptone-physiological solution (PPS) [0.1 % (w/v) bacteriological peptone (Oxoid) and 0.85 % (w/v) NaCl]. Sample dilutions were incubated at 30 and 37 °C under aerobic and anaerobic conditions on MRS5 agar containing 0.1 g cycloheximide l⁻¹ (Meroth *et al.*, 2003). Colonies were picked, checked for bacteriological purity and stored in Microbank tubes (Pro-Lab Diagnostics) at –80 °C. Isolates LMG 23699^T, LMG 23701, LMG 23702 and R-30103 were cultured in an aerobic atmosphere at 30 °C. Isolates LMG 23701 and LMG 23702 originate from a wheat sourdough produced in the province of Namur, Belgium, that was sampled in 2002. Isolates LMG 23699^T and R-30103 were recovered from a wheat sourdough sampled in 2004 and 2005, respectively. The latter sourdough was produced in the province of Oost-Vlaanderen, Belgium. Gram-staining, cell morphology, catalase activity and all further experiments were performed with cells cultivated for 24 h on MRS5 medium and incubation at 30 °C under aerobic conditions, unless otherwise indicated.

The original collection of sourdough isolates obtained during the biodiversity studies was initially screened by means of rep-PCR fingerprinting. For rep-PCR analysis, total DNA was extracted from single colonies grown on MRS5 agar by alkaline lysis. A small portion of one colony was resuspended in 20 µl lysis buffer (2.5 ml 10 % SDS, 5.0 ml 1 M NaOH, 92.5 ml Milli-Q water). The mixture was heated at 95 °C for 15 min, cooled immediately on ice and after a short centrifugation at high speed, 180 µl sterile Milli-Q water was added. Subsequently, the mixture was centrifuged at 13 000 g for 5 min and stored at –20 °C. If the alkaline lysis method was not able to produce good quality rep-PCR profiles, a phenol/chloroform method was used, as described by Gevers *et al.* (2001). The rep-PCR oligonucleotide primer used in this study was (GTG)₅ (5'-GTGGTGGTGGTGGTG-3') (Versalovic *et al.*, 1994). The conditions for amplification and gel electrophoresis were as reported by Versalovic *et al.* (1994). The (GTG)₅-PCR profiles were visualized after staining with ethidium bromide under UV light, followed by digital image capturing using a CCD camera. The resulting fingerprints were analysed using the BioNumerics V4.0 software package (Applied Maths). Isolates were tentatively assigned to a given species when high similarities between the (GTG)₅-PCR fingerprints of isolates and reference strains of typical sourdough species were obtained. A separate (GTG)₅-PCR

cluster containing 11 sourdough isolates from two different bakeries could not be allocated to any species in the (GTG)₅-PCR reference framework (data not shown). Four isolates from this cluster that produced a unique (GTG)₅-PCR band pattern, i.e. LMG 23699^T, LMG 23701, LMG 23702 and R-30103, were selected for further polyphasic characterization.

The taxonomic position of the four selected sourdough isolates was first investigated using *pheS* gene sequence analysis (Naser *et al.*, 2005). Genomic DNA was extracted as described by Gevers *et al.* (2001). The primers for *pheS* sequencing were PheS-21-F and PheS-23-R, using amplification conditions and sequencing reactions as described by Naser *et al.* (2005). Sequences were imported into the BioNumerics V4.0 software, aligned and compared using the neighbour-joining method with available sequences of nearly all recognized *Lactobacillus* species. All four isolates constituted a separate sub-branch in the *Lactobacillus plantarum* group, showing sequence similarities below 90 % with the other members of the group (Fig. 1). Isolates LMG 23699^T, LMG 23701 and R-30103 shared 100 % sequence similarity, whereas isolate LMG 23702 showed 90.2 % *pheS* sequence similarity with the other three sourdough isolates. Interspecies gaps in lactobacilli normally exceed 7 % (S. M. Naser, P. S. R. Dawyndt, B. Hoste, D. Gevers, K. Vandemeulebroecke, I. Cleenwerck, M. Vancanneyt & J. Swings, unpublished), which suggested that the four sourdough isolates represented one or possibly two new *Lactobacillus* species.

The phylogenetic position of all isolates was determined by complete 16S rRNA gene sequence analysis. Genomic DNA was extracted as described above. 16S rRNA gene amplification, purification and sequencing were performed as described previously (Vancanneyt *et al.*, 2006). The obtained sequences (continuous stretches of 1519 bp) were deposited in, and aligned and clustered with sequences from the EMBL database. A phylogenetic tree was constructed using the neighbour-joining method and the BioNumerics V4.0 software package. Unknown bases were discarded for analysis. The statistical reliability of the tree was evaluated by bootstrap analysis of 500 replicates and the tree topology was confirmed using maximum-parsimony and maximum-likelihood. 16S rRNA gene sequence analysis indicated that the four sourdough isolates clearly belong to the *Lactobacillus plantarum* group. Isolate LMG 23699^T is most closely positioned to *Lactobacillus nantensis* (LMG 23510^T), *Lactobacillus mindensis* (LMG 21932^T) and *Lactobacillus farciminis* (LMG 9200^T), showing 16S rRNA gene sequence similarities of 98.4, 98.9 and 99.3 %, respectively (Fig. 2). All four new isolates shared more than 99.5 % 16S rRNA gene sequence similarity, which contrasted with the separate position of isolate LMG 23702 revealed by *pheS* sequence analysis.

The sourdough LAB isolates were also investigated using SDS-PAGE of cellular proteins. Extraction and gel electrophoresis were performed according to the method

Fig. 1. Neighbour-joining tree showing the relationships of *pheS* sequence data of the sourdough isolates *Lactobacillus crustorum* LMG 23699^T, LMG 23701, LMG 23702 and R-30103 with reference strains of the *Lactobacillus plantarum* group. *Lactobacillus sanfranciscensis* (LMG 16002^T) was used as outgroup. Bootstrap values (based on 500 replications) at or above 50 % are indicated at the branch points. Bar, 10 substitutions per 100 nt positions.

described by Pot *et al.* (1994) for Gram-positive bacteria. Densitometric digitization of patterns was performed using an LKB 2202 Ultrascan Laser Densitometer. Normalization of densitometric traces was performed using the GELCOMP version 4.2 software (Applied Maths). Numerical analysis with profiles available in an extensive in-house database was performed using the Pearson product moment correlation coefficient and the unweighted pair-group method using arithmetic averages (UPGMA) with BioNumerics V4.0 software. Among the protein profiles of the four sourdough

isolates, notable differences were observed in the position and intensity of the dominant bands situated in a molecular mass range of 25–35 kDa. Numerical analysis of the protein profiles, omitting the latter zone, resulted in the delineation of one distinct branch for the four sourdough isolates (see supplementary Fig. S1 with the online version of this paper). Between isolates LMG 23699^T and R-30103, only minor differences were observed in the intensity of dominant bands which could suggest that both isolates are subcultures of a diverging clone.

Fig. 2. Phylogenetic tree derived from 16S rRNA gene sequence analysis showing the phylogenetic position of *Lactobacillus crustorum* LMG 23699^T, LMG 23701, LMG 23702 and R-30103 among related taxa. The tree was generated by the neighbour-joining method and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LMG 6901^T) was used as outgroup. Bootstrap values (based on 500 replications) at or above 50% are indicated at the branch points. Bar, 1 substitution per 100 nt positions.

Further genotypic characterization and differentiation of the four new isolates was performed using amplified fragment length polymorphism (AFLP) fingerprinting of whole genomes. Extraction and purification of total genomic DNA was done as described above. AFLP analysis was performed according to the protocol of Thompson *et al.* (2001) with several modifications. Total DNA was digested with *Eco*RI and *Taq*I restriction enzymes. Fragments were amplified using the primers E01 (5'-GACTGCGTACCAATTCA-3')

and T01 (5'-CGATGAGTCCTGACCGAA-3'). The resulting electrophoretic profiles were normalized using the GeneScan 3.1 software (Applied), and tables of peaks, containing fragments of 50–536 bp, were transferred into the BioNumerics V4.0 software. AFLP patterns were compared with profiles of LAB reference taxa using the Dice coefficient and UPGMA linkage. Cluster analysis of the AFLP band patterns confirmed the unique taxonomic position of isolates LMG 23699^T, LMG 23701, LMG 23702

and R-30103. Also, we observed that the AFLP pattern of isolate LMG 23702 was the most divergent compared to the other three sourdough isolates. Although isolates LMG 23699^T and R-30103 displayed highly similar fingerprints, AFLP analysis allowed strain differentiation among the four new isolates and thus excluded clonal relatedness (Fig. 3).

For the determination of G + C content, DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). Genomic DNA was extracted as described above. The nucleoside mixture was separated by HPLC using a Waters SymmetryShield C8 column maintained at 37 °C. The solvent was 0.02 M (NH₄)H₂PO₄ (pH 4.0) with 1.5 % acetonitrile. Non-methylated λ -phage DNA (Sigma) was used as the calibration reference. The DNA G + C contents of the strains LMG 23699^T, LMG 23701, LMG 23702 and R-30103 were 35, 36, 35 and 35 mol%, respectively. These values are within the range reported for the *Lactobacillus plantarum* group (34–46 mol%) (Ehrmann *et al.*, 2003).

DNA–DNA hybridizations were performed between strains LMG 23699^T, LMG 23701, LMG 23702 and R-30103 and the type strains of the seven most closely related species, i.e. *Lactobacillus farciminis*, *Lactobacillus nantensis*, *Lactobacillus mindensis*, *Lactobacillus kimchii*, *Lactobacillus paralimentarius*, *Lactobacillus alimentarius* and *Lactobacillus versmoldensis* (Figs 1 and 2). DNA was extracted from 0.75–1.25 g (wet wt) cells using the protocol described by Gevers *et al.* (2001) with the following modifications. Volumes were increased tenfold for large-scale application. After the addition of 20 % SDS and glass beads, cells were mixed for 30 s. Subsequently, 16.5 ml TE buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) and 5 ml 5 M NaCl were added, followed by 10 min incubation at 65 °C. The chloroform/isoamylalcohol extraction, and ethanol and RNase treatment were performed as described by Marmur (1961). DNA–DNA hybridizations were performed with biotin-labelled probes in microplate wells (Ezaki *et al.*, 1989), using an HTS7000 Bio Assay Reader (Perkin Elmer)

for the fluorescence measurements. The hybridization temperature was 34 °C in the presence of 50 % formamide. Reciprocal experiments were performed for every pair of strains and standard deviation values ranged from 0.3 to 12. DNA–DNA binding values between strain LMG 23699^T and the type strain of *Lactobacillus alimentarius* (LMG 9187^T), *Lactobacillus farciminis* (LMG 9200^T), *Lactobacillus nantensis* (LMG 23510^T), *Lactobacillus paralimentarius* (LMG 19152^T), *Lactobacillus mindensis* (LMG 21932^T), *Lactobacillus kimchii* (LMG 19822^T) and *Lactobacillus versmoldensis* (LMG 21929^T) were 35, 33, 31, 31, 30, 26 and 14 %, respectively. These values are well below the threshold of 70 % suggested for species delineation (Stackebrandt & Goebel, 1994), indicating that LMG 23699^T represents a novel species in the genus *Lactobacillus*. Furthermore, DNA–DNA hybridizations were performed between strain LMG 23699^T and strains LMG 23701, LMG 23702 and R-30103. Hybridization values were above the 70 % threshold (range 72–96 %), indicating that all strains belong to the same species. DNA–DNA binding values were lowest between strain LMG 23702 and strains LMG 23699^T, LMG 23701 and R-30103 (range 72–77 %), which is in line with the slightly aberrant position of the former strain in *pheS* sequencing and AFLP analysis (Figs 1 and 3). Despite this, polyphasic taxonomic evidence collectively indicates that strain LMG 23702 is sufficiently close to the other three sourdough strains to be considered a member of the same species.

Growth characteristics of strains LMG 23699^T, LMG 23701, LMG 23702 and R-30103 were determined in MRS broth (pH 5.4) (de Man *et al.*, 1960). Growth was tested at 15 and 45 °C and in the presence of 5, 6 and 7 % NaCl. Aerobic and anaerobic growth and production of gas from 2 % glucose and 2 % gluconate in MRS-broth (pH 5.4, without triammonium citrate) was investigated. Arginine hydrolysis was tested in a medium containing 0.5 % tryptone, 0.5 % yeast extract, 0.3 % L-arginine, 0.05 % glucose and 0.2 % K₂HPO₄ (pH 7.0), with methyl orange as indicator. The isomeric type of lactate was determined enzymically (R-Biopharm).

Fig. 3. Cluster analysis of digitized AFLP band patterns of *L. crustorum* and closest phylogenetic relatives. The corresponding dendrogram was constructed from the UPGMA linkage of Dice coefficients.

The carbohydrate fermentation patterns of the strains were determined using the API 50 CHL system (bioMérieux), following the manufacturer's instructions with strains cultivated at 37 °C. A detailed phenotypic description is given below and characteristics differentiating the new species from its closest relatives *Lactobacillus farciminis*, *Lactobacillus nantensis* and *Lactobacillus mindensis* are summarized in Table 1.

Considering all evidence from the polyphasic taxonomic study, a novel species, *Lactobacillus crustorum* sp. nov., is proposed for strains LMG 23699^T, LMG 23701, LMG 23702 and R-30103.

Description of *Lactobacillus crustorum* sp. nov.

Lactobacillus crustorum (crus.tor'um. L. gen. pl. n. *crustorum*, of breads/cakes).

Cells are Gram-positive, catalase-negative, non-motile and non-spore-forming. The rod-shaped cells occur singly or in pairs, are 2–15 µm in length and 0.5–1 µm wide. After 24 h

incubation on MRS5 agar, colonies are beige, slightly irregular, convex with a slightly rough surface and approximately 0.5–2 mm in diameter. The cells grow well in liquid or solid MRS under aerobic or anaerobic conditions. The strains grow at 15 °C in the presence of 5, 6 and 7 % NaCl and at 45 °C. All strains produce more than 90 % of the L-lactate isomer and glucose is metabolized homofermentatively. Ammonium is not produced from arginine. No gas is produced from glucose or gluconate. All strains produce acid from galactose, glucose, fructose, mannose, *N*-acetylglucosamine, aesculin and salicin, but not from glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methyl-D-xyloside, sorbose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, melibiose, sucrose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate. Acid production from rhamnose, amygdalin, arbutin, cellobiose, maltose, lactose, trehalose, gentiobiose and D-tagatose is strain-dependent. The DNA G + C content is 35–36 mol%.

The type strain, LMG 23699^T (=CCUG 53174^T), was isolated from an artisan wheat sourdough produced in the province of Oost-Vlaanderen, Belgium.

Table 1. Phenotypic characteristics of *Lactobacillus crustorum* strains LMG 23699^T, LMG 23701, LMG 23702 and R-30103 and reference strains of their closest relatives

Strains: 1, *Lactobacillus nantensis* LMG 23510^T (data from Valcheva *et al.*, 2006); 2, *Lactobacillus farciminis* LMG 9200^T (data from Reuter, 1983); 3, *Lactobacillus mindensis* LMG 21932^T (data from Ehrmann *et al.*, 2003); 4, *Lactobacillus crustorum* sp. nov. LMG 23699^T; 5, *Lactobacillus crustorum* sp. nov. LMG 23701; 6, *Lactobacillus crustorum* sp. nov. LMG 23702; 7, *Lactobacillus crustorum* sp. nov. R-30103. +, Positive after 48 h incubation; (+), positive after 5 days incubation; –, negative; W, weakly positive.

Production of acid from:	1	2	3	4	5	6	7
Ribose	W	–	–	–	–	–	–
Galactose	+	+	–	+	+	+	+
Rhamnose	–	–	–	–	–	(+)	–
Mannitol	+	–	–	–	–	–	–
Sorbitol	+	–	–	–	–	–	–
α-Methyl-D-mannoside	+	–	–	–	–	–	–
N-Acetylglucosamine	+	–	+	+	+	+	+
Amygdalin	+	–	W	+	–	(+)	+
Arbutin	+	–	–	(+)	–	–	+
Aesculin	+	+	–	+	+	+	+
Cellobiose	+	+	+	+	–	+	–
Maltose	+	W	+	+	–	(+)	+
Lactose	+	+	–	+	+	+	+
Melibiose	+	–	–	–	–	–	–
Sucrose	+	+	–	–	–	–	–
Trehalose	+	W	–	+	(+)	(+)	+
Melezitose	–	–	–	–	–	–	–
Raffinose	+	W	–	–	–	–	–
Gentiobiose	+	–	–	+	+	+	+
D-Tagatose	+	–	–	+	+	+	+

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