Reclassification of *Lactobacillus brevis* strains LMG 11494 and LMG 11984 as *Lactobacillus parabrevis* sp. nov.

Marc Vancanneyt,[1] Sabri M. Naser,[1,2] Katrien Engelbeen,[1] Marjan De Wachter,[1] Roel Van der Meulen,[3] Ilse Cleenwerck,[1] Bart Hoste,[1] Luc De Vuyst[3] and Jean Swings[1,2]

Correspondence
Marc Vancanneyt
Marc.Vancanneyt@UGent.be

[1,2]BCCM/LMG Bacteria Collection[1] and Laboratory of Microbiology[2], Faculty of Sciences, Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium

[3]Research Group of Industrial Microbiology, Fermentation Technology and Downstream Processing (IMDO), Department of Applied Biological Sciences, Vrije Universiteit Brussel (VUB), Pleinlaan 2, B-1050 Brussels, Belgium

A polyphasic study revealed taxonomic heterogeneity among reference strains of the species *Lactobacillus brevis*. Representative strains of *L. brevis* and related taxa were investigated by partial sequence analysis of the housekeeping gene encoding the alpha-subunit of phenylalanyl-tRNA synthase (*pheS*). Species-specific clusters were delineated for all taxa studied except for two *L. brevis* strains, LMG 11494 and LMG 11984, respectively isolated from cheese and wheat, which occupied a distinct position. Their phylogenetic affiliation was determined using 16S rRNA gene sequence analysis and it was found that both strains (with 99·9 % gene sequence similarity between them) belonged to the *Lactobacillus buchneri* group, with nearest neighbours *Lactobacillus hammesii* and *L. brevis* (gene sequence similarities of 99·2 and 98·1 %, respectively). Further genotypic and phenotypic studies, including fluorescent amplified fragment length polymorphism, DNA–DNA hybridization and DNA G+C content, clearly demonstrated that the two strains represent a single novel taxon for which the name *Lactobacillus parabrevis* sp. nov. is proposed (type strain LMG 11984^T=ATCC 53295^T).

Lactobacillus brevis strains are frequently isolated from the spoilage microbiota in wine and beer fermentations, but also occur in fermented foods and feed such as sourdoughs, sour starch, cheeses, olives and silage (Stiles & Holzapfel, 1997). Identification of strains of *L. brevis* using conventional phenotypic methods often leads to ambiguous results (Kandler & Weiss, 1986; Pot *et al.*, 1994). Molecular approaches have proved to be much more reliable (Vogel *et al.*, 1994; Sohier *et al.*, 1999; Guarneri *et al.*, 2001) and have enabled '*L. brevis*-like' isolates to be assigned to novel taxa such as *Lactobacillus acidifarinae*, *Lactobacillus hammesii*, *Lactobacillus spicheri* and *Lactobacillus zymae* (Meroth *et al.*, 2004; Valcheva *et al.*, 2005; Vancanneyt *et al.*, 2005). In the present study, reference strains of *L. brevis* available from the BCCM/LMG Bacteria Collection (http://www.belspo.be/bccm/lmg.htm) were screened genotypically and the results demonstrated that two strains, LMG 11494 and LMG 11984, occupied a distinct position. Further genomic and phenotypic research revealed that these strains represent a single novel species.

Strain LMG 11494 (=NCFB 1058) was isolated from farmhouse red Cheshire cheese and was originally deposited in the NCFB culture collection as *L. brevis* by A. Hayward in 1957. Strain LMG 11984 (=ATCC 53295) was isolated from wheat and deposited in the ATCC as *L. brevis* (originally named *Sporolactobacillus* sp.) by M. Spiller in 1992. It is a patent strain used for the production of leavening barm (Spiller, 1987). Both strains and related reference strains were cultivated and maintained on de Man, Rogosa and Sharpe (MRS) agar medium (pH 6·5; de Man *et al.*, 1960) and incubated at 30 °C for 24–48 h, unless otherwise indicated.

Sequence analysis of the phenylalanyl-tRNA synthase alpha-subunit (*pheS*) housekeeping gene has been proved to be a robust approach for the identification of enterococci (Naser *et al.*, 2005a). Furthermore, the method is an excellent tool for delineating novel taxa (Naser *et al.*, 2005b; Švec *et al.*,

Abbreviation: FAFLP, fluorescent amplified fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 11494 and LMG 11984^T are AM158250 and AM158249, respectively.

2005a, b). In the present study, this methodology was applied to lactobacilli of the *Lactobacillus buchneri* species group. The primer sequences, amplification conditions and sequencing reactions performed were as described by Naser *et al.* (2005a). As found previously for enterococci, a species-specific grouping was obtained, as all *Lactobacillus* species studied formed distinct clusters (data not shown). Only two *L. brevis* strains, LMG 11494 and LMG 11984, showed an aberrant position. The neighbour-joining tree depicted in Fig. 1 (based upon comparison of partial sequences of 309 bp) revealed the relatedness between strains LMG 11494 and LMG 11984 and type strains of related taxa and showed that the two strains under study constituted a distinct cluster with a gene sequence similarity of 97 %. Nearest neighbours were the type strains of *L. acidifarinae*, *L. hammesii*, *L. spicheri* and *L. zymae*, with sequence similarities in a significantly lower range of 85–87 %. *L. brevis* and other taxa of the *L. buchneri* species group were more distantly related, with sequence similarities below 82 %.

The phylogenetic position of strains LMG 11494 and LMG 11984 was further determined by complete 16S rRNA gene sequence analysis. DNA for 16S rRNA gene sequencing was prepared by heating one or two colonies at 95 °C for 15 min in 20 µl lysis buffer containing 0·25 % (w/v) SDS and 0·05 M NaOH. Following lysis, 180 µl distilled water was added to the lysate. 16S rRNA genes were amplified using oligonucleotide primers complementary to highly conserved regions of bacterial 16S rRNA genes. The forward primer was 5′-AGAGTTTGATCCTGGCTCAG-3′ (hybridizing at positions 8–27, according to the *Escherichia coli* numbering system) and the reverse primer was 5′-AAGGAGGTGATCCAGCCGCA-3′ (hybridizing at positions 1541–1522). PCR products were purified by using a NucleoFast 96 PCR clean-up kit (Macherey Nagel). Sequencing reactions were performed by using a BigDye Terminator Cycle sequencing kit (Applied Biosystems) and purified by using a Montage SEQ_{96} sequencing reaction cleanup kit (Millipore). Sequencing was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The eight sequencing primers used are listed in Coenye *et al.* (1999). Sequence assembly was performed using the AUTOASSEMBLER program (Applied Biosystems). Sequences were aligned with sequences retrieved from GenBank using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic analyses and bootstrap analysis (500 replicates) were subsequently performed using the BioNumerics 4.01 software package (Applied Maths). A phylogenetic tree was constructed using the neighbour-joining method (Fig. 2; Saitou & Nei, 1987) and unknown bases were discarded for the analyses. Comparison of the newly determined complete sequences for strains LMG 11494 and LMG 11984 (continuous stretches of 1518 bp) revealed a sequence similarity of 99·9 %. The tree topology obtained with the neighbour-joining method was evaluated and confirmed by maximum parsimony analysis using BioNumerics (data not shown). Comparison with deposited sequences available in the EMBL database classified strains LMG 11494 and LMG 11984 as part of the *L. buchneri* group (Schleifer & Ludwig, 1995) with the nearest neighbours (>97 % sequence similarity) *L. hammesii* and *L. brevis*, showing sequence similarities of 99·2 and 98·1 %, respectively.

Strains LMG 11494 and LMG 11984 were further screened using PAGE of whole-cell proteins. Whole-cell protein extracts were prepared and SDS-PAGE was performed as described by Pot *et al.* (1994). Densitometric analysis, normalization and interpolation of protein profiles and a

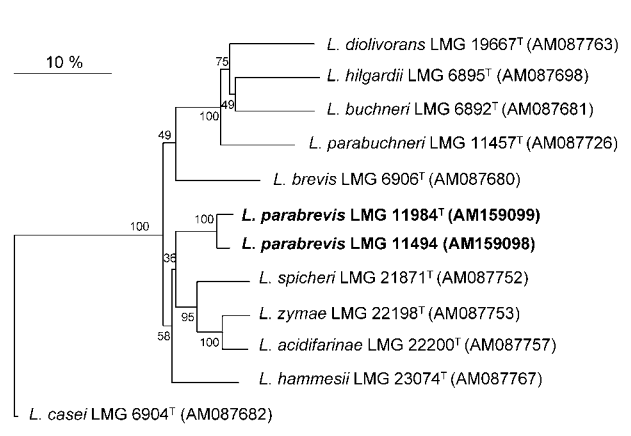

Fig. 1. Neighbour-joining tree based on the partial *pheS* gene sequences of *L. parabrevis* and other reference species belonging to the *L. buchneri* group. Bootstrap percentages after 500 simulations are shown. *Lactobacillus casei* LMG 6904^{T} was included as an outgroup. Bar, 10 % difference in nucleotide sequence.

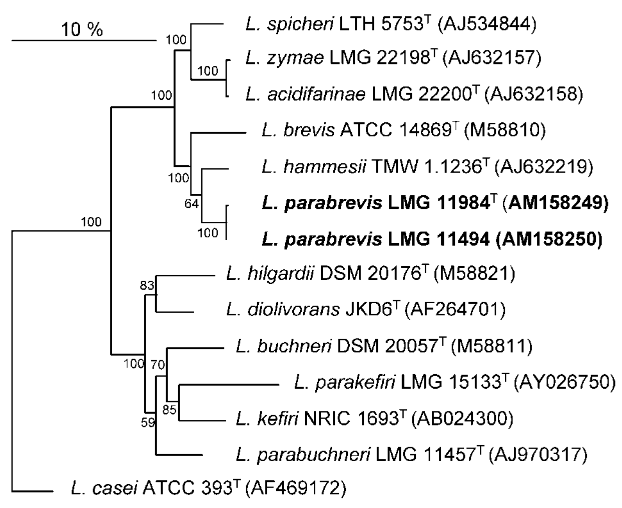

Fig. 2. Distance matrix tree showing the phylogenetic relationships of *L. parabrevis* and other reference species belonging to the *L. buchneri* group, based on 16S rRNA gene sequence comparisons. *L. casei* ATCC 393^{T} was used as the outgroup. Bootstrap probability values (percentages of 500 tree replications) are indicated at branch-points. Bar, 10 % difference in nucleotide sequence.

numerical analysis were performed by using the GELCOMPAR software package, versions 3.1 and 4.0, respectively (Applied Maths). After comparison with an in-house database containing profiles of nearly all recognized lactic acid bacteria, the two strains could not be distinguished from representatives of *L. brevis*. Phylogenetically related species, such as *L. acidifarinae*, *L. hammesii*, *L. spicheri* and *L. zymae*, occupied distinct positions (data not shown). Although this approach is very valuable for species identification of most lactic acid bacteria, some closely related species are not distinguished by this method, as has been shown previously within the *Lactobacillus plantarum* species group (Torriani *et al.*, 2001) and the *Lactobacillus acidophilus* species group (Gancheva *et al.*, 1999).

Strains LMG 11494 and LMG 11984 and a representative set of reference strains were investigated by a genotypic screening approach using fluorescent amplified fragment length polymorphism (FAFLP) fingerprinting of whole genomes. FAFLP fingerprinting was performed as described by Thompson *et al.* (2001) with the following modifications: *Eco*RI/*Taq*I was used as the restriction enzyme combination and the primer combination E01/T01 (both having an adenosine extension at the 3′-end) was applied for selective PCR. The resulting electrophoretic patterns were tracked and normalized using GENESCAN 3.1 software (Applera). Normalized tables of peaks, containing fragments of 50–536 bp, were transferred into the BioNumerics software package, version 3.5, and the computer-generated fingerprints were added to an existing database of FAFLP fingerprints of lactic acid bacteria held at the BCCM/LMG Bacteria Collection. For numerical analysis, the region between the 75 and 500 bp bands of the internal standard were used. Similarity was calculated using the Dice coefficient and clustering was performed using the UPGMA algorithm. The dendrogram obtained from the analysis (Fig. 3) confirmed the distinct taxonomic position of strains LMG 11494 and LMG 11984.

DNA G+C contents were determined for strains LMG 11494 and LMG 11984. DNA was extracted from 0·75–1·25 g (wet weight) using the protocol described by Gevers *et al.* (2001), using a combination of glass beads and enzymes, but with the following modifications. Volumes were increased tenfold for application on a large scale. SDS-treated cells were vortexed with beads for 30 s. After addition of 16·5 ml buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8·0) and 5 ml 5 M NaCl and gentle mixing, the suspension was incubated at 65 °C for 10 min. Subsequent chloroform/isoamylalcohol extraction, precipitation, spooling of DNA on a glass rod, washing with ethanol and RNase treatment were performed as described by Marmur (1961). For determination of the DNA G+C content, DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture was then separated by HPLC using a SymmetryShield RP8 column (Waters) maintained at 37 °C. The solvent was 0·02 M $(NH_4)H_2PO_4$ (pH 4·0) with 1·5 % acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. DNA G+C contents of strains LMG 11494 and LMG 11984 were 49 mol%. This value is lower than the value of 52·6 mol% determined for the type strain of *L. hammesii* and significantly higher than the value of 46 mol% determined for the type strain of *L. brevis* (Valcheva *et al.*, 2005).

DNA–DNA hybridizations were performed between strains LMG 11494 and LMG 11984 and the type strains of *L. brevis* and *L. hammesii* (DNA was prepared as described above). Strain LMG 11984 was further hybridized with the type strains of *L. acidifarinae*, *L. spicheri* and *L. zymae*. The microplate method was used as described by Ezaki *et al.* (1989) and Goris *et al.* (1998), using a HTS7000 Bio Assay Reader (Perkin Elmer) for fluorescence measurements. Biotinylated single-stranded DNA (ssDNA) was hybridized with unlabelled ssDNA, which was bound non-covalently to microplate wells. Hybridizations were performed at 40 °C in

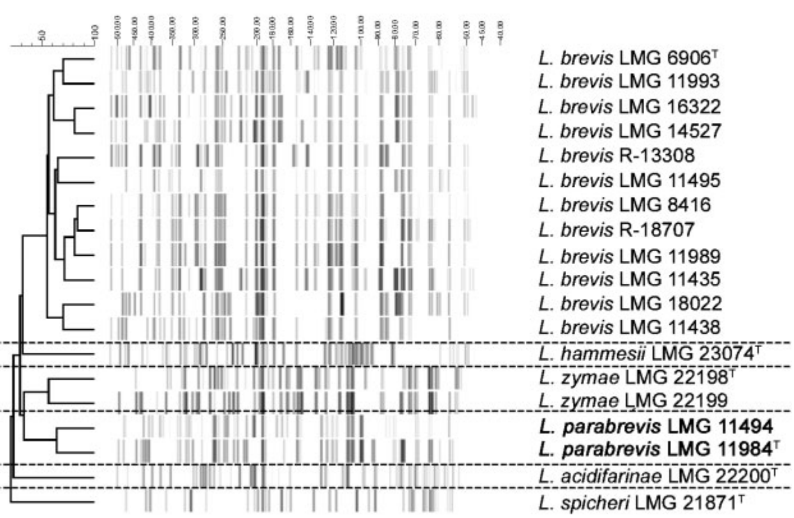

Fig. 3. FAFLP patterns and corresponding dendrogram, derived from the UPGMA linkage of Dice coefficients (expressed as a percentage value for convenience) of *L. parabrevis* strains and related reference strains.

hybridization mixture (2 × SSC, 5 × Denhardt's solution, 2·5 % dextran sulphate, 50 % formamide, 100 μg denatured salmon sperm DNA ml^{-1} and 1·25 μg biotinylated probe DNA ml^{-1}). The DNA–DNA relatedness percentages presented are mean values based on four independent hybridization experiments. Reciprocal reactions (e.g. A × B and B × A) were performed and are also considered as independent hybridization experiments. Strains LMG 11494 and LMG 11984 displayed hybridization values of 35 and 40 %, respectively, with the type strain of *L. hammesii* and 7 and 8 %, respectively, with the type strain of *L. brevis*. Strain LMG 11984 yielded values in the range of 5–18 % with the type strains of *L. acidifarinae*, *L. spicheri* and *L. zymae*. The DNA–DNA hybridization value between strains LMG 11494 and LMG 11984 was 90 %, indicating a separate species status for the latter strains.

Growth characteristics and colony morphology were investigated on MRS agar after 24 h incubation at 37 °C under aerobic conditions and are given below in the species description.

Conventional biochemical tests were performed as described by Vancanneyt *et al.* (2005) and carbohydrate fermentation tests were carried out using API 50 CHL galleries following the manufacturer's instructions (bioMérieux). Metabolites from glucose were lactate, acetate and ethanol, as determined by HPLC (Waters). Isomers of D- and L-lactate were determined enzymically (R-Biopharm) and were in the ratio of 4 : 6 for strains LMG 11494 and LMG 11984. Strains LMG 11494 and LMG 11984 were differentiated from their nearest neighbour *L. hammesii* by their ability to deaminate arginine and inability to produce acid from aesculin or mannitol (Valcheva *et al.*, 2005). Features that differentiated the two strains from *L. brevis* were the abilities to produce acid from D-arabitol and methyl β-xyloside (Vancanneyt *et al.*, 2005). Phenotypic data determined in the present study and by Vancanneyt *et al.* (2005) also revealed distinct features of the two novel strains which separate them from the more distantly related taxa *L. acidifarinae*, *L. spicheri* and *L. zymae*.

The overall results of the present study enable strains LMG 11494 and LMG 11984 to be assigned as representatives of a novel species, for which the name *Lactobacillus parabrevis* sp. nov. is proposed.

Description of *Lactobacillus parabrevis* sp. nov.

Lactobacillus parabrevis (pa.ra.bre′vis. Gr. prep. *para* like; L. masc. adj. *brevis* referring to the specific epithet of *Lactobacillus brevis*; N.L. masc. adj. *parabrevis* brevis-like, referring to *L. brevis*).

Cells are rod-shaped, occurring singly or in pairs and in chains, 1·5–5 μm in length and 0·9 μm wide, Gram-positive, catalase-negative, non-spore-forming and non-motile. After 24 h, colonies are beige, circular with a smooth surface and approximately 1 mm in diameter. Growth occurs at 15 °C, but not at 45 °C. Growth occurs at 6 % NaCl. Facultatively anaerobic and produces DL-lactic acid heterofermentatively with acetic acid and ethanol as other metabolites from glucose. Gas is produced from glucose and gluconate. Arginine is deaminated. All strains produce acid from L-arabinose, D-arabitol, gluconate, *N*-acetylglucosamine, D-glucose, D-fructose, maltose, ribose, D-xylose and methyl β-xyloside. All strains test negative for acid production from D-arabinose, L-arabitol, adonitol, amygdalin, arbutin, cellobiose, dulcitol, aesculin, erythritol, D- and L-fucose, β-gentiobiose, 2- and 5-ketogluconate, glycerol, glycogen, inositol, inulin, D-lyxose, mannitol, D-mannose, methyl α-D-mannoside, melezitose, melibiose, D-raffinose, rhamnose, sucrose, salicin, starch, sorbitol, L-sorbose, D-tagatose, trehalose, D-turanose, xylitol and L-xylose. Acid production from galactose, methyl α-D-glucoside and lactose is strain-dependent. The G + C content of DNA is 49 mol%.

The type strain, LMG 11984^T (= ATCC 53295^T), was isolated from wheat. A reference strain, LMG 11494 (= NCFB 1058), has also been isolated from cheese.

Acknowledgements

This research was supported by the Prime Minister's Services - Federal Office for Scientific, Technical and Cultural Affairs, Belgium, in particular the action to stimulate the use of and cooperation with the Belgian Coordinated Collections of Microorganisms (contract no. C3/00/17). S. M. N. acknowledges a PhD scholarship from the Ministry of Education and Higher Education.

References

Coenye, T., Falsen, E., Vancanneyt, M., Hoste, B., Govan, J. R. W., Kersters, K. & Vandamme, P. (1999). Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. *Int J Syst Bacteriol* **49**, 405–413.

de Man, J. C., Rogosa, M. & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**, 130–135.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Gancheva, A., Pot, B., Vanhonacker, K., Hoste, B. & Kersters, K. (1999). A polyphasic approach towards the identification of strains belonging to *Lactobacillus acidophilus* and related species. *Syst Appl Microbiol* **22**, 573–585.

Gevers, D., Huys, G. & Swings, J. (2001). Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol Lett* **205**, 31–36.

Goris, J., Suzuki, K., De Vos, P., Nakase, T. & Kersters, K. (1998). Evaluation of a microplate DNA-DNA hybridization method compared with the initial renaturation method. *Can J Microbiol* **44**, 1148–1153.

Guarneri, T., Rossetti, L. & Giraffa, G. (2001). Rapid identification of *Lactobacillus brevis* using the polymerase chain reaction. *Lett Appl Microbiol* **33**, 377–381.

Kandler, O. & Weiss, N. (1986). Genus *Lactobacillus* Beijerinck 1901, 212^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1209–1234. Edited by P. H. A. Sneath, N. S. Mair & J. G. Holt. Baltimore: Williams & Wilkins.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Meroth, C. B., Hammes, W. P. & Hertel, C. (2004). Characterisation of the microbiota of rice sourdoughs and description of *Lactobacillus spicheri* sp. nov. *Syst Appl Microbiol* **27**, 151–159.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Naser, S. M., Thompson, F. L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M. & Swings, J. (2005a). Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. *Microbiology* **151**, 2141–2150.

Naser, S. M., Vancanneyt, M., De Graef, E. & 8 other authors (2005b). *Enterococcus canintestini* sp. nov., from faecal samples of healthy dogs. *Int J Syst Evol Microbiol* **55**, 2177–2182.

Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: Wiley.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Schleifer, K. H. & Ludwig, W. (1995). Phylogeny of the genus *Lactobacillus* and related genera. *Syst Appl Microbiol* **18**, 461–467.

Sohier, D., Coulon, J. & Lonvaud-Funel, A. (1999). Molecular identification of *Lactobacillus hilgardii* and genetic relatedness with *Lactobacillus brevis*. *Int J Syst Bacteriol* **49**, 1075–1081.

Spiller, M. A. (1987). *A mixture of a Lactobacillus brevis and Saccharomyces dairensis for preparing leavening barm*. US Patent 4,666,719. 19 May 1987, US Patent Office.

Stiles, M. E. & Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *Int J Food Microbiol* **36**, 1–29.

Švec, P., Vancanneyt, M., Devriese, L. A., Naser, S. M., Snauwaert, C., Lefebvre, K., Hoste, B. & Swings, J. (2005a). *Enterococcus aquimarinus* sp. nov., isolated from sea water. *Int J Syst Evol Microbiol* **55**, 2183–2187.

Švec, P., Vancanneyt, M., Koort, J., Naser, S. M., Hoste, B., Vihavainen, E., Vandamme, P., Swings, J. & Björkroth, J. (2005b). *Enterococcus devriesei* sp. nov., associated with animal sources. *Int J Syst Evol Microbiol* **55**, 2479–2484.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Thompson, F. L., Hoste, B., Vandemeulebroecke, K. & Swings, J. (2001). Genomic diversity amongst *Vibrio* isolates from different sources determined by fluorescent amplified fragment length polymorphism. *Syst Appl Microbiol* **24**, 520–538.

Torriani, S., Clementi, F., Vancanneyt, M., Hoste, B., Dellaglio, F. & Kersters, K. (2001). Differentiation of *Lactobacillus plantarum*, *L. pentosus* and *L. paraplantarum* species by RAPD-PCR and AFLP. *Syst Appl Microbiol* **24**, 554–560.

Valcheva, R., Korakli, M., Onno, B., Prévost, H., Ivanova, I., Ehrmann, M. A., Dousset, X., Gänzle, M. G. & Vogel, R. F. (2005). *Lactobacillus hammesii* sp. nov., isolated from French sourdough. *Int J Syst Evol Microbiol* **55**, 763–767.

Vancanneyt, M., Neysens, P., De Wachter, M. & 8 other authors (2005). *Lactobacillus acidifarinae* sp. nov. and *Lactobacillus zymae* sp. nov., from wheat sourdoughs. *Int J Syst Evol Microbiol* **55**, 615–620.

Vogel, R. F., Bocker, G., Stolz, P. & 7 other authors (1994). Identification of lactobacilli from sourdough and description of *Lactobacillus pontis* sp. nov. *Int J Syst Bacteriol* **44**, 223–229.