Reclassification of *Lactobacillus ferintoshensis* as a later heterotypic synonym of *Lactobacillus parabuchneri*

Marc Vancanneyt, Katrien Engelbeen, Marjan De Wachter, Katrien Vandemeulebroecke, Ilse Cleenwerck and Jean Swings

Correspondence

Marc Vancanneyt
Marc.Vancanneyt@rug.ac.be

BCCM/LMG Bacteria Collection, Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium

Lactobacillus ferintoshensis has recently been described as a novel species, distinct from its close phylogenetic neighbours *Lactobacillus buchneri*, *Lactobacillus kefir* and *Lactobacillus hilgardii*. Two highly related species with validly published names, *Lactobacillus parakefir* and *Lactobacillus parabuchneri*, were not considered in the study due to the lack of 16S rRNA gene sequence data at that time. Since the publication of the study, the sequences have become available and have revealed that *L. ferintoshensis* and *L. parabuchneri* share 99.7% 16S rRNA gene sequence similarity. Further genomic and phenotypic data, derived from fluorescent amplified fragment length polymorphism, DNA–DNA hybridization and API 50 CHL analyses, have demonstrated that the species are synonymous.

Lactobacillus ferintoshensis, isolated from Scotch malt whisky distilleries, was described by Simpson *et al.* (2001) based on three strains, the type strain R7-84^T (=LMG 22038^T = CIP 106749^T), strain R7-9 and strain R15-103. The type strain was compared with *Lactobacillus buchneri*, *Lactobacillus kefir* and *Lactobacillus hilgardii*, its nearest phylogenetic neighbours, showing 98.4, 98.1 and 97.3% 16S rRNA gene sequence similarities, respectively. DNA–DNA hybridizations and further phenotypic characterization demonstrated that *L. ferintoshensis* represented a novel species. In their study, however, the authors did not include two related species, *Lactobacillus parabuchneri* (Farrow *et al.*, 1988) and *Lactobacillus parakefir* (Takizawa *et al.*, 1994), which were species with validly published names at that time but lacked publicly available 16S rRNA gene sequence data. Subsequent sequence analysis demonstrated a very high similarity of 99.7% between *L. ferintoshensis* and *L. parabuchneri* (see below). In the present study, the relatedness between the latter two species was studied and indicated that there was synonymy between the two taxa.

Two reference strains of *L. ferintoshensis*, LMG 22038^T and strain R15-103, both isolated from a whisky distillery, were included in this study. The latter strain R15-103 (assigned as LMG 22462) was received from F. G. Priest (Heriot-Watt

University, Edinburgh, UK), the corresponding author for the *L. ferintoshensis* paper. For *L. parabuchneri*, strains LMG 11457^T and LMG 11973, isolated from human saliva and brewery yeast, respectively, were selected for further comparative study. *L. ferintoshensis* strains were cultivated and maintained on de Man, Rogosa and Sharpe (MRS) medium supplemented with 0.5% maltose and incubated aerobically at 28 °C. *L. parabuchneri* strains and other reference strains studied were cultivated and maintained on MRS medium and incubated anaerobically at 37 °C, unless indicated otherwise.

The phylogenetic relatedness between the type strains of *L. ferintoshensis* and *L. parabuchneri* was investigated. For the latter strain, only a partial sequence of 1219 bp was available at GenBank/EMBL and so a nearly complete 16S rRNA gene sequence was determined in this study. Genomic DNA from LMG 11457^T was prepared according to the protocol of Niemann *et al.* (1997). 16S rRNA gene amplification, purification and sequencing was performed as described by Vancanneyt *et al.* (2004) with the following modifications: PCR-amplified 16S rRNAs 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 using a Montage SEQ₉₆ sequencing reaction clean-up kit (Millipore). Electrophoresis of sequence reaction products was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sequence assembly was carried out with the AutoAssembler program (Applied Biosystems). The 16S rRNA gene sequence of *L. parabuchneri* LMG 11457^T, a continuous stretch of 1527 bp, was

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Abbreviation: FAFLP, fluorescent amplified fragment length polymorphism.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Lactobacillus parabuchneri* LMG 11457^T is AJ970317.

determined and was aligned with the sequence of the type strain of *L. ferintoshensis*, retrieved from GenBank/EMBL. A phylogenetic tree was constructed by the neighbour-joining method using the BioNumerics software package, version 3.5 (Applied Maths). Unknown bases were discarded for the analyses. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the neighbour-joining tree using 500 bootstrap resamplings of the data (Fig. 1). A comparison of the sequences of the type strains of *L. ferintoshensis* and *L. parabuchneri* revealed 99.7% sequence similarity.

L. ferintoshensis LMG 22038^T and LMG 22462 were investigated using fluorescent amplified fragment length polymorphism (FAFLP) fingerprinting of whole genomes and the patterns obtained were compared with those of related taxa. FAFLP fingerprinting was performed as described by Thompson *et al.* (2001) with the following modifications: *EcoRI*/*TaqI* 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 (Applied). Normalized tables of peaks, containing fragments of 50 to 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 at the BCCM/LMG Bacteria Collection. For numerical analysis, data between the 75 and 500 bp bands of the internal standard were used. Clustering was performed using a Dice coefficient and the UPGMA algorithm. The FAFLP fingerprints of the two strains were compared with reference profiles of lactic acid bacteria taxa as currently available from the BCCM/LMG database. FAFLP analysis revealed a high similarity between *L.*

Fig. 1. Distance matrix tree showing the phylogenetic relationships of *L. ferintoshensis* and *L. parabuchneri* and other reference species belonging to the *L. buchneri* group, based on 16S rRNA gene sequence comparisons. *Lactobacillus brevis* was used as the outgroup and bootstrap values (percentages of 500 tree replications) are indicated at branch points. Bar, 1% sequence divergence.

ferintoshensis and *L. parabuchneri*. Fig. 2 shows a dendrogram in which *L. ferintoshensis* and *L. parabuchneri* strains were found to be grouped in a single cluster and separated from related reference species.

DNA G + C content was determined for the *L. ferintoshensis* and *L. parabuchneri* strains. DNA was extracted from 0.75–1.25 g (wet weight) according to the protocol described by Gevers *et al.* (2001), using a combination of glass beads and enzymes (for LMG 11973, mutanolysin was added to a final concentration of 750 U ml⁻¹) but with the following modifications. Volumes were increased by 10 × for use on a large scale. The SDS-treated cells were vortexed with beads for 30 s. After the addition of 16.5 ml buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) and 5 ml 5 M NaCl, followed by gentle shaking, the suspension was incubated at 65 °C for 10 min. Subsequent chloroform/isoamylalcohol extraction, precipitation, spooling of DNA onto a glass rod, washing with ethanol and RNase treatment were performed as described by Marmur (1961). After RNase treatment, proteinase K (1 mg ml⁻¹; Merck) was added to the mixture. For the determination of DNA G + C content, DNA was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture was then separated by HPLC using a Waters SymmetryShield C8 column maintained at a temperature of 37 °C. The solvent used comprised 0.02 M (NH₄)₂HPO₄ (pH 4.0) with 1.5% acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. DNA G + C content of all four strains was 43 mol%. This value was close to the value of 44 mol% determined for the type strain of *L. parabuchneri* (Farrow *et al.*, 1998).

DNA–DNA hybridizations were performed between *L. ferintoshensis* LMG 22038^T and LMG 22462, and between *L. parabuchneri* LMG 11457^T and LMG 11973 (DNA was prepared as described above). The microplate method was used as described by Ezaki *et al.* (1989) and Goris *et al.* (1998), using an HTS7000 Bio Assay Reader (Perkin Elmer) for fluorescence measurements. Biotinylated single-stranded (ss) DNA was hybridized with unlabelled ssDNA, which was bound non-covalently to microplate wells. Hybridizations were performed at 39 °C in a hybridization mixture containing 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 DNA probe ml⁻¹. The DNA–DNA hybridization level between the four strains was in a range of 77 to 93%; indicating that the two species actually constitute a single species. Strain LMG 22462 which showed a high binding level (73%) with the type strain of the species under non-stringent conditions and intermediate levels (44%) under stringent conditions in Simpson *et al.* (2001), demonstrated a binding level of 93% to the type strain of *L. ferintoshensis* in our experiments.

Growth characteristics and biochemical features were investigated with cells grown on MRS agar supplemented with maltose after 24 h incubation at 28 °C under aerobic conditions. Carbohydrate fermentation tests were carried out

Fig. 2. FAFLP patterns and corresponding dendrogram, derived from the UPGMA linkage of Dice coefficients (expressed as a percentage value for convenience) of *L. ferintoshensis* and *L. parabuchneri* strains and related reference strains.

using the API 50 CHL galleries according the manufacturer's instructions (bioMérieux). Acid is produced from L-arabinose, D-fructose, galactose, gluconate, D-glucose, maltose, melezitose, melibiose, ribose and sucrose. No acidification of adonitol, amygdalin, D-arabinose, D-arabitol, L-arabitol, arbutin, cellobiose, dulcitol, erythritol, aesculin, D-fucose, L-fucose, β -gentiobiose, 2-ketogluconate, N-acetylglucosamine, methyl α -D-glucoside, glycerol, glycogen, inositol, inulin, lactose, mannitol, D-mannose, methyl α -D-mannoside, D-lyxose, D-raffinose, rhamnose, salicin, sorbitol, L-sorbose, starch, D-tagatose, trehalose, D-turanose, xylitol, D-xylose, L-xylose or methyl β -xyloside are observed. Acid production from 5-ketogluconate is variable and is only weakly positive for the type strain of *L. parabuchneri*. These data are in agreement with the data of Farrow *et al.* (1988), except for acid formation of D-raffinose, which could not be confirmed.

The data from the present study show that *L. ferintoshensis* strains belong to the species *L. parabuchneri*. Consequently, *L. ferintoshensis* Simpson *et al.* 2002 is a later heterotypic synonym of *L. parabuchneri* Farrow *et al.* 1989.

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