Reclassification of *Lactobacillus thermotolerans* Niamsup *et al.* 2003 as a later synonym of *Lactobacillus ingluviei* Baele *et al.* 2003

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The relatedness of the species *Lactobacillus ingluviei* and *Lactobacillus thermotolerans* was investigated by comparing partial sequences of the 16S rRNA gene (99.9% similarity over 1504 bp), the *hsp60* gene (98.8% similarity over 954 bp) and the *recA* gene (98.5% similarity over 452 bp) and by determining DNA–DNA binding levels (79 ± 3%) and genomic DNA G + C contents (50 and 49 mol%, respectively). These data, in addition to their similar biochemical characteristics, suggest that the two taxa constitute a single species. According to Rules 38 and 42 of the *Bacteriological Code*, they should be united under the name *Lactobacillus ingluviei*, with the name *Lactobacillus thermotolerans* as a later heterotypic synonym.

Lactobacillus ingluviei Baele *et al.* 2003 was described as follows on the basis of the characteristics of seven strains isolated from pigeon crops and intestines. Cells are Gram-positive (rapidly decolourizing in the Gram-stain procedure), non-motile, very short, plump rods that mostly occur singly or in pairs. Better growth is obtained at 42 °C than at 37 or 30 °C; no growth occurs at 25 °C. Acid is produced from L-arabinose, D-fructose, methyl β-xyloside, ribose, sucrose and D-xylose; fermentation of D-glucose, maltose, D-mannose, D-raffinose, gluconate and aesculin is variable. The DNA G + C content is 49 mol%. This species, based on 16S rRNA gene sequence analysis, belongs to the *Lactobacillus reuteri* phylogenetic group and highest sequences similarities were obtained to the sequences of *Lactobacillus fermentum* and *Lactobacillus mucosae*.

Lactobacillus thermotolerans Niamsup *et al.* 2003 was described on the basis of morphological, physiological and biochemical characteristics of five thermotolerant strains isolated from chicken faeces in Thailand. Cells are Gram-positive, non-motile, non-spore-forming, catalase-negative rods, 1 × 2–3 µm in size, which occur singly, in pairs or as

short chains. Obligately heterofermentative, they produce both D- and L-lactic acid isomers. Growth is observed up to 50 °C, but not at 15 °C (the optimum temperature is 42 °C). *meso*-Diaminopimelic acid is not present in the cell wall and the DNA G + C content of the type strain is 50.5 mol%.

A comparative sequence analysis based on 16S rRNA-encoding genes obtained from GenBank (accession numbers AF333975 for *L. ingluviei* KR3^T and AF317702 for *L. thermotolerans* G 35^T) revealed very high similarity; only two nucleotides were found to be different (99.87% similarity over a total of 1504 bp). Moreover, the physiological traits reported by the authors, the habitats from which these bacteria have been isolated and the observation that the two species were described in the same year (and no reciprocal comparison was therefore made) suggest the necessity of clarifying their taxonomic relationship with additional genetic approaches.

L. ingluviei LMG 20380^T and *L. thermotolerans* DSM 14792^T (= LMG 22056^T) were grown in MRS broth at 37 °C under anaerobiosis and bacterial cultures were checked for purity.

In order to look at the difference between the two species, two protein-encoding genes, *hsp60* and *recA*, were sequenced and compared. It has already been reported that sequences of protein-encoding genes have higher discriminatory power than 16S rRNA gene sequences (Felis *et al.*, 2001; Rosselló-Mora & Amann, 2001). Sequence comparison of

The GenBank/EMBL/DDBJ accession numbers for the partial *hsp60* gene sequences of *L. ingluviei* LMG 20380^T and *L. thermotolerans* DSM 14792^T are respectively AJ621722 and AJ621723 and the accession numbers for the partial *recA* gene sequences of *L. ingluviei* LMG 20380^T and *L. thermotolerans* DSM 14792^T are respectively AJ972423 and AJ621677.

these genes has proven to be helpful to describe the relationships among closely related taxa (Felis *et al.*, 2001; Torriani *et al.*, 2001; Dellaglio *et al.*, 2004, 2005; Bringel *et al.*, 2005), although no threshold of sequence similarity for these protein-encoding genes has yet been proposed.

DNA of type strains LMG 20380^T and DSM 14792^T was extracted following the procedure of Marmur (1961). Partial *hsp60* gene sequences were amplified using two degenerate primers, cpn-f and cpn-r, as described by Dellaglio *et al.* (2005) and amplification products of about 1000 bp were obtained. Sequencing reactions were performed at the Bio-molecular Research Centre (BMR), University of Padua, Italy, with the primers used during the PCR. Sequences of 954 bp, representing about the 57% of the complete gene (considering the complete *hsp60* gene sequences NT01LP0651 and NT01LJ0492 of the genome sequences of *Lactobacillus plantarum* WCSF1 and *Lactobacillus johnsonii* NCC 533, respectively), were obtained for the two type strains and compared. A total of 11 nucleotides were found to be different.

Partial *recA* gene sequences were obtained using the degenerate primers recEXT-f and recEXT1-r as described by Dellaglio *et al.* (2005). Thermal conditions were optimized to improve amplification efficiency as follows: an initial denaturation of 5 min at 94 °C, 35 cycles of 45 s at 94 °C, 120 s at 50 °C and 105 s at 72 °C and a final extension at 72 °C for 7 min. PCR products of 750 bp, about half of the complete gene sequence considering the complete *recA* gene of *L. plantarum* WCSF1 and *L. johnsonii* NCC 533, were obtained for the *L. ingluviei* and *L. thermotolerans* type strains. Sequence comparison revealed seven differences from a total of 452 bp.

In order to clarify the taxonomic status of the two species further, a DNA–DNA hybridization test was performed between the two type strains *L. ingluviei* LMG 20380^T and *L. thermotolerans* LMG 22056^T and their G + C contents were redetermined. For strain LMG 20380^T, DNA was extracted from 0.75–1.25 g wet weight of cells using the protocol described by Pitcher *et al.* (1989) with the following modifications: the washed cell pellet was resuspended and lysed in a buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) containing RNase (200 µg ml⁻¹; Sigma), mutanolysin (100 U ml⁻¹; Sigma) and lysozyme (25 mg ml⁻¹; SERVA) for 1 h at 37 °C. Before addition of GES reagent (5 M guanidine thiocyanate, 100 mM EDTA, 0.5% Sarkosyl in water), proteinase K (200 µg ml⁻¹; Merck) was added to the mixture for 15 min. For strain LMG 22056^T, DNA was extracted from 0.75–1.25 g wet weight of cells by 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 and gentle shaking with 16.5 ml buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) and 5 ml 5 M NaCl, the suspension was incubated at 65 °C for 10 min. Subsequent chloroform/isoamyl alcohol extraction, precipitation,

spooling of DNA on a glass rod, washing with ethanol and RNase treatment was performed as described by Marmur (1961). For DNA–DNA hybridizations, 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 DNA was hybridized with unlabelled single-stranded DNA, which was bound non-covalently to microplate wells. Hybridizations were performed at 41 °C in hybridization mixture (2 × SSC, 5 × Denhardt's solution, 2.5% dextran sulphate, 50% formamide, 100 µg denatured salmon sperm DNA ml⁻¹, 1.25 µg biotinylated probe DNA ml⁻¹). The percentage DNA relatedness presented is a mean value, based on four independent hybridization experiments. Reciprocal reactions (i.e. A × B and B × A) were performed and are also considered as independent hybridization experiments.

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 Waters SymmetryShield C8 column maintained at a temperature of 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.

Results showed that the type strains of *L. ingluviei* and *L. thermotolerans* had a binding level of 79 ± 3%; consequently, as currently delineated (Rosselló-Mora & Amann, 2001; Stackebrandt & Goebel, 1994), the two species constitute a single taxonomic unit. The G + C contents of the type strains were 50 mol% for *L. ingluviei* LMG 20380^T and 49 mol% for *L. thermotolerans* DSM 14792^T.

Considering the high value of total DNA hybridization obtained, the possibility of describing two subspecies was evaluated to group strains of the two former species. Therefore, a few fermentation tests were performed: data reported in the original species descriptions were compared and it was found that melibiose was fermented by *L. thermotolerans* but not by *L. ingluviei*. Both species are able to metabolize D-fructose and not lactose. These experiments were repeated by growing *L. ingluviei* LMG 20380^T and *L. thermotolerans* DSM 14792^T at 30 °C for 24–48 h in phenol red medium broth with lactose, melibiose or fructose (1% final concentration) added as the sole carbon source. Lactose and fructose were used as negative and positive controls, respectively. The two type strains yield identical metabolic profiles, as both metabolized fructose, but neither fermented melibiose or lactose, in contrast to the description of the species *L. thermotolerans* (Niamsup *et al.*, 2003).

Although the two species were isolated from slightly different environments (pigeons and chickens) and from very distant places (Europe and Asia) and display sequences of two protein-encoding genes that are not identical, the delineation of intraspecific clusters was not considered in this study. Therefore it is proposed that the two species

Lactobacillus ingluviei and *Lactobacillus thermotolerans* should be united under the same name. According to the rules of priority (Rules 38 and 42 of the *Bacteriological Code*; Lapage *et al.*, 1992), the name *Lactobacillus ingluviei* should be retained with its type strain KR3^T (=CCUG 45722^T = DSM 15946^T = JCM 12531^T = LMG 20380^T). Consequently, strains of *L. thermotolerans* should be reclassified as belonging to *L. ingluviei*. Due to the very similar traits reported in the descriptions of the two species, an emended description of *L. ingluviei* is not considered necessary. The species *L. ingluviei* belongs to the *L. reuteri* phylogenetic group within the genus *Lactobacillus* as delineated by Hammes & Hertel (2003).

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