Reclassification of *Leuconostoc argentinum* as a later synonym of *Leuconostoc lactis*

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Leuconostoc argentinum, *Leuconostoc lactis* and ten related strains from Romanian dairy products formed a single cluster, clearly separated from other *Leuconostoc* species, after numerical analysis of repetitive extragenic palindromic-PCR patterns, whole-cell protein profiles (SDS-PAGE) and fluorescent amplified fragment length polymorphism (FAFLP) band patterns. 16S rRNA gene sequence analysis confirmed a very high similarity between both type strains and representative dairy isolates (>99.6%). DNA–DNA hybridization experiments revealed high relatedness values between the type strains of *L. argentinum* and *L. lactis* and between these strains and representative Romanian strains. These data and the lack of phenotypic distinctive characteristics demonstrate that *L. argentinum* and *L. lactis* are synonymous.

Leuconostoc argentinum was described as a separate species, distinct from *Leuconostoc lactis*, based on total soluble cell protein analysis, DNA–DNA hybridization experiments and phenotypic data (Dicks *et al.*, 1993). No 16S rRNA gene sequence data were available at that time. In a recent study on the biodiversity of lactic acid bacteria from traditional Romanian dairy products, a number of strains could not be assigned to either *L. argentinum* or *L. lactis* after numerical analysis of repetitive extragenic palindromic-PCR (REP-PCR) patterns (unpublished results). 16S rRNA gene sequence data (available at EMBL) for members of the genus *Leuconostoc* show that *L. argentinum* and *L. lactis* are nearest neighbours (>99.6% sequence similarity) and constitute a separate

phylogenetic branch together with a third species, *Leuconostoc citreum*. A polyphasic study was performed to investigate the taxonomic relatedness between *L. argentinum* and *L. lactis* strains and the Romanian dairy isolates.

Strains studied included (i) two reference strains of *L. lactis*, LMG 8894^T and LMG 7940, isolated from milk and cheese, respectively; (ii) the type strain of *L. argentinum* LMG 18543^T, from Argentinian raw milk; and (iii) ten Romanian isolates: strains LMG 22631, LMG 22635 and R-19870 from raw cows' milk, strains LMG 22614, LMG 22627, LMG 22628 and LMG 22660 from sour cream and strains LMG 22591, LMG 22650 and R-21065 from cheese. All strains used in this study were obtained from the BCCM/LMG Bacteria Collection (<http://www.belspo.be/bccm/lmg.htm>) and were cultivated and maintained on de Man, Rogosa and Sharpe (MRS) agar medium (de Man *et al.*, 1960) and were incubated aerobically at 30 °C, unless otherwise indicated.

REP-PCR fingerprinting with the (GTG)₅ primer was initially applied as a deduplication approach for all Romanian lactic acid bacteria isolates collected from dairy products (unpublished results). Subsequent analysis of the patterns was performed using the BioNumerics software version 4.0 according to Gevers *et al.* (2001). The (GTG)₅-PCR

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Abbreviations: FAFLP, fluorescent amplified fragment length polymorphism; REP-PCR, repetitive extragenic palindromic-PCR.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Leuconostoc lactis* LMG 22650 and R-21065 are AJ970316 and AJ937759, respectively.

A dendrogram based on (GTG)₅-PCR fingerprints and a table detailing the phenotypic features of *Leuconostoc lactis* strains are available as supplementary material in IJSEM Online.

fingerprints obtained were compared with an in-house database covering reference strains of all *Leuconostoc* species. *L. lactis*, *L. argentinum* and the ten Romanian strains displayed similar fingerprints. Cluster analysis grouped them into a single cluster, clearly separated from fingerprints obtained for the other *Leuconostoc* species (data not shown). (GTG)₅-PCR fingerprint patterns obtained from the strains investigated in this study and revealing their differentiation from the phylogenetically closest species, *Leuconostoc citreum*, are available in Supplementary Fig. S1 in IJSEM Online.

All strains of the *L. argentinum*–*L. lactis* cluster were further screened using SDS-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 numerical analysis were performed by using GELCOMP software, versions 3.1 and 4.0, respectively (Applied Maths). A dendrogram confirmed that the *L. lactis*, *L. argentinum* and Romanian isolates constitute a homogeneous and separate cluster that is distinct from *L. citreum* (Fig. 1) and other *Leuconostoc* species (data not shown).

Because of its higher taxonomic resolution compared with SDS-PAGE of proteins (Gancheva *et al.*, 1999; Torriani *et al.*, 2001), all reference strains and novel isolates were also investigated 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: *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 the GENESCAN 3.1 software (Applied). 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 at the BCCM/LMG Bacteria Collection. For numerical analysis, data between the 75 and

500 bp bands of the internal standard were used. Similarity was calculated using the Dice coefficient and clustering was done using the UPGMA algorithm. The FAFLP fingerprints of all strains were compared with reference profiles of lactic acid bacteria taxa as currently available in the database. FAFLP analysis revealed a high similarity between *L. lactis*, *L. argentinum* and the Romanian isolates. Fig. 2 shows a dendrogram in which the latter strains grouped in a single cluster separated from the related reference species *L. citreum*.

The phylogenetic position of the Romanian isolates R-21065 and LMG 22650 was determined by complete 16S rRNA gene sequence analysis performed as described by Vancanneyt *et al.* (2004) with the following modifications. PCR-amplified 16S rRNA genes were purified by using a NucleoFast 96 PCR clean-up kit (Macherey-Nagel). Sequencing reactions were purified using a Montage SEQ₉₆ sequencing reaction clean-up kit (Millipore). Sample preparation was assisted using a Genesis Workstation 200 (Tecan). Electrophoresis of sequence reaction products was performed by using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequences (continuous stretches of 1502 bp) and sequences of strains retrieved from EMBL were aligned and a comparison of the newly determined complete sequences revealed a sequence similarity of 99.8%. A comparison with sequences available in the EMBL database classified the strains with nearest neighbours *L. lactis* and *L. argentinum* (sequence similarities above 99.8 and 99.6%, respectively). Within the genus *Leuconostoc*, these taxa and *L. citreum* occupy a distinct branch.

DNA G+C contents were determined for *L. argentinum* LMG 18543^T, *L. lactis* LMG 8894^T and LMG 7940, *L. citreum* LMG 9849^T and strains LMG 22627, LMG 22628, LMG 22635 and R-21065. DNA was extracted from 0.75–1.25 g (wet weight) of cells according to the protocol described by Gevers *et al.* (2001) using a combination of glass beads and enzymes, but with the following modifications. Volumes were increased ten-fold for application on a large scale. Vortexing with beads of the SDS-treated cells was performed for 30 s. After addition of 16.5 ml buffer (10 mM Tris/HCl,

Fig. 1. Protein profiles and corresponding dendrogram, derived from UPGMA linkage of correlation coefficients (*r*, expressed as a percentage value for convenience) of *L. argentinum*, *L. lactis*, *L. citreum* and related strains from Romanian dairy products.

Fig. 2. FAFLP patterns and corresponding dendrogram, derived from the UPGMA linkage of Dice coefficients (expressed as a percentage value for convenience) of *L. argentinum*, *L. lactis*, *L. citreum* and related strains isolated from Romanian dairy products.

100 mM EDTA, pH 8.0) and 5 ml 5 M NaCl and gentle shaking, 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 was performed as described by Marmur (1961). After the RNase treatment, proteinase K (1 mg ml⁻¹; VWR International) was added to the mixture. 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 37 °C. The solvent was 0.02 M (NH₄)H₂PO₄ (pH 4.0) with 1.5% acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. DNA G+C contents of *L. argentinum* and *L. lactis* were 42 and 43 mol%, respectively. These values were close to the value of 40.5 mol% which was determined for the type strain of *L. argentinum* (Dicks *et al.*, 1993) and also in the range of 43–45 mol% determined for *L. lactis* (Garvie, 1986). Similar values of 42 mol% were obtained for all the Romanian isolates. For the type strain of *L. citreum*, the value of 39 mol% determined in this study was similar to the value of 38–40 mol% described in the literature (Farrow *et al.*, 1989).

DNA–DNA hybridizations were performed between *L. argentinum* LMG 18543^T, *L. lactis* LMG 8894^T and LMG 7940, *L. citreum* LMG 9849^T and strains LMG 22627, LMG 22628, LMG 22635 and R-21065. 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 DNA (ssDNA) was hybridized with unlabelled ssDNA, which was bound non-covalently to microplate wells. Hybridizations were performed at 37 °C in a hybridization mixture containing 2 × SSC, 5 × Denhardt's solution, 2.5% dextran sulfate, 50% formamide, 100 µg denatured salmon sperm DNA ml⁻¹ and 1.25 µg biotinylated probe DNA ml⁻¹. The DNA–DNA hybridization value between the type strain of *L. argentinum* and the type strain of *L. lactis* was 81%, indicating that they constitute a single species. The value

determined in this study was significantly higher than that given in Dicks *et al.* (1993). Four Romanian isolates, LMG 22627, LMG 22628, LMG 22635 and R-21065 could be assigned to this single species with values ranging from 72 to 94%. *L. citreum* was clearly distinct, with DNA–DNA relatedness values below 23% with all strains tested.

Biochemical features were investigated by both conventional fermentation tests and the standard commercial identification system API 50 CH (bioMérieux). The inoculum size was 1% (v/v) and was prepared by growing a single colony of each strain for 24 h, centrifuging 1 ml culture at 8000 r.p.m. for 2 min and resuspending the cells in 1 ml sterile physiological solution (0.9% NaCl w/v). The culture assay medium, prepared in the laboratory or provided by the manufacturer (for the conventional and API tests, respectively), had the following composition (l⁻¹): 10 g polypeptone, 5 g yeast extract, 2 g dipotassium phosphate, 5 g sodium acetate, 2 g diammonium citrate, 0.2 g magnesium sulfate, 0.05 g manganese sulfate, 1 ml Tween 80, pH 6.7–7.1. For the conventional biochemical tests, carbohydrates (sucrose, D-lactose, D-fructose, L-arabinose, D-xylose, D-maltose, D-trehalose, D-galactose and salicin) were added to a final concentration of 5 g l⁻¹. Cultures were incubated at 37 °C for 48 h. Results of conventional and API tests were congruent and are summarized in Supplementary Table S1 in IJSEM Online. No characteristic phenotype profile was found for *L. lactis* or *L. argentinum* as suggested in the literature (Dicks *et al.*, 1993), although the differentiation proposed by the latter authors was only valid for a minority of the strains tested. Most of the Romanian isolates showed atypical features compared with the type strains *L. argentinum* LMG 18543^T and *L. lactis* LMG 8894^T and most had fermentation patterns more similar to *L. lactis* LMG 7940. The inability of the strains to ferment D-galactose, except for *L. lactis* LMG 8894^T, is in disagreement with previous reports. In addition, strain *L. lactis* LMG 8894^T did not ferment fructose, an activity regarded as positive for all *L. lactis* strains (Garvie, 1986). *Leuconostoc* sp. LMG 22660 was the only strain that hydrolysed aesculin. The same strain also exhibited an atypical fermentation pattern for some other carbohydrates (see Supplementary Table S1 in IJSEM

Online). *Leuconostoc* sp. LMG 22650 and LMG 22660 were unique in their ability to ferment D-xylose. These observations indicate considerable variability in the physiological characterization of strains belonging to the *L. lactis* group and identification is only reliable after confirmation with molecular techniques. The inability to differentiate *L. lactis* from *L. argentinum* using phenotypic features was noted in the original description of *L. argentinum* by Dicks *et al.* (1993).

The data from the present study show that the type strain of *L. argentinum* and all the Romanian dairy isolates are members of the species *L. lactis*. Consequently, it is proposed that *L. argentinum* Dicks *et al.* 1993 be considered as a later synonym of *L. lactis* Garvie 1960.

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