Leuconostoc rapi sp. nov., isolated from sous-vide-cooked rutabaga

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A Gram-stain-positive, ovoid, lactic acid bacterium, strain LMG 27676ᵀ, was isolated from a spoiled sous-vide-cooked rutabaga. 16S rRNA gene sequence analysis indicated that the novel strain belongs to the genus Leuconostoc, with Leuconostoc kimchii and Leuconostoc miyukkimchii as the nearest neighbours (99.1 and 98.8 % 16S rRNA gene sequence similarity towards the type strain, respectively). Phylogenetic analysis of the 16S rRNA gene, multilocus sequence analysis of the pheS, rpoA and atpA genes, and biochemical and genotypic characteristics allowed differentiation of strain LMG 27676ᵀ from all established species of the genus Leuconostoc. Strain LMG 27676ᵀ (≡R-50029ᵀ=MHB 277₁=DSM 27776₁) therefore represents the type strain of a novel species, for which the name Leuconostoc rapi sp. nov. is proposed.

The genus Leuconostoc, which at the time of writing comprises 12 species, belongs phylogenetically to the phylum Firmicutes, class Bacilli, order Lactobacillales. Cells of members of the genus Leuconostoc are Gram-stain-positive, small, regular, ovoid cocci, occurring in pairs or short chains. The cells are non-motile and do not form spores. Species of the genus Leuconostoc are considered psychrophilic mesophiles with optimal growth at 14–30 °C. The temperature limits for growth vary among species and strains, ranging from 1–10 °C to 30–40 °C (Säde, 2011). In 2008, Leuconostoc fructosum, Leuconostoc durionis, Leuconostoc fisculeum and Leuconostoc pseudofisculeum were reclassified on the basis of phylogenetic, physiological and morphological differences into the genus Fructobacillus (Endo & Okada, 2008).

Species of the genus Leuconostoc are associated with the fermentation of vegetables (Di Cagno et al., 2013). Recently described species of the genus Leuconostoc originating from fermented vegetables include Leuconostoc inhae and Leuconostoc kimchii from kimchi, a Korean vegetable product (Kim et al., 2000, 2003), and Leuconostoc miyukkimchii from fermented miyukkimchii made of a brown algae (Undaria pinnatifida), a regional kimchi in Korea (Lee et al., 2012). Other novel species include Leuconostoc palmae from palm wine, an alcoholic beverage produced from the sap of various palm tree species (Ehrmann et al., 2009), and Leuconostoc holzapelii, from Ethiopian coffee fermentation (De Bruyne et al., 2007). The occurrence of leuconostocs including Leuconostoc gelidum, Leuconostoc gascomitatum (which were recently reclassified as L. gelidum subsp. gelidum and L. gelidum subsp. gascomitatum, respectively; Rahkila et al., 2014), Leuconostoc citreum and Leuconostoc mesenteroides, in other vegetables has also been reported (Garg et al., 1990; García-Gimeno & Zurera-Cosano, 1997; Lyhs et al., 2004; Vihavainen et al., 2008).

Strain LMG 27676ᵀ was isolated from packaged sous-vide-cooked rutabaga (Brassica napobrassica) during an investigation of the spoilage microbiota in sous-vide-cooked vegetables in Finland. For microbiological analyses, a 10 g rutabaga sample was weighed aseptically into 90 ml buffered peptone water (LabM) in a sterile plastic bag and
then blended for 60 s using a Dilumat automatic system (AES laboratoire, bioMérieux). Tenfold serial dilutions were used for microbiological analyses. The number of lactic acid bacteria was determined on MRS agar (Oxoid) supplemented with sorbic acid (pH 6.4). All plates were incubated under anaerobic conditions at 20 °C for 7 days. Three of the isolates obtained, LMG 27676 T, L. pseudomesenteroides DSM 5578 T (99.1 %) and L. miyukkimchii M2 T (98.8 %), exhibited identical randomly amplified polymorphic DNA fingerprints as determined using the primers RAPD-270 (5′-TGCCGCAGG-3′) and RAPD-272 (5′-AGCGGGCCCA-3′) (Mahenthiralingam et al., 1996) (data not shown).

Nearly complete 16S rRNA gene sequence analysis for strain LMG 27676 T was performed as described by Vancanneyt et al. (2004). PCR products were purified using a Nucleofast 96 PCR clean up membrane system (Machery-Nagel). The sequencing primers were those listed by Coenye et al. (1999) and the fragments obtained were cleaned with a BigDye XTerminator Purification kit (Applied Biosystems, Life Technologies), according to the manufacturer’s instructions. The mothur software package and the corresponding SILVA reference alignment were used to align the 16S rRNA gene sequences of strain LMG 27676 T (1507 bp) with sequences of type strains of all established species of the genus Leuconostoc. These aligned sequences were imported into MEGA6 (Tamura et al., 2013) and analysed using the neighbour-joining, maximum-likelihood and maximum-parsimony methods. The statistical reliability of tree topologies was evaluated by bootstrapping analysis based on 1000 replicates. The neighbour-joining and the maximum-parsimony trees revealed topologies similar to those obtained in the maximum-likelihood tree (Fig. 1). 16S rRNA gene sequence similarities were calculated using MEGA6 (Tamura et al., 2013) and the closest relatives of strain LMG 27676 T were L. kimchii IH25 T (99.1 %) and L. miyukkimchii M2 T (98.8 %). Lower sequence similarities (<98.6 %) were found with other members of the genus Leuconostoc.

Sequence analysis of the housekeeping genes encoding phenylalanyl-tRNA synthase alpha subunit (pheS), RNA

Fig. 1. Maximum-likelihood tree of the 16S rRNA gene sequence of strain LMG 27676 T with those of all type strains of the genus Leuconostoc. The evolutionary history was inferred by using the maximum-likelihood method based on the Kimura two-parameter model (Kimura, 1980). The tree with the highest log likelihood (-3107.7404) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter=0.0500)]. The rate variation model allowed for some sites to be evolutionarily invariable (+I, 12.2611 % sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 nucleotide sequences. Codon positions included were noncoding. All positions with less than 95 % site coverage were eliminated. That is, fewer than 5 % alignment gaps, missing data and ambiguous bases were allowed at any position. There were a total of 1379 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).
polymerase alpha subunit (rpoA) and the alpha subunit of ATP synthase (atpA) correlates with a species delineation as determined by DNA–DNA hybridization in the genus Leuconostoc (De Bruyne et al., 2007). Therefore, pheS, rpoA and atpA gene sequences of strains LMG 27676T, L. kimchii KCTC 2386T and L. miyukkimchii LMG 27695T, for which no pheS, rpoA or atpA sequences were available, were determined as described previously (De Bruyne et al., 2007), except that sequencing reactions were purified using the BigDye Terminator purification kit as described above. The pheS, rpoA and atpA gene sequences of the closest relatives (Leuconostoc carnosum LMG 23898T, L. inhae LMG 22919T, L. gelidum subsp. gasicomitatum LMG 18811T, L. gelidum subsp. gelidum LMG 18297T and L. gelidum subsp. aenigmaticum LMG 27840T) of strain LMG 27676T were available from previous studies (De Bruyne et al., 2007; Rahkila et al., 2014).

The software package MEGA6 (Tamura et al., 2013) was used to align the translated concatenated gene sequences and to analyse the nucleotide sequences as mentioned above. The maximum-parsimony and neighbour-joining trees for both analyses (not shown) revealed topologies similar to those obtained in a phylogenetic tree reconstructed using the maximum-likelihood approach (Fig. 2). The concatenated pheS, rpoA and atpA gene sequences of strain LMG 27676T revealed high similarity to the concatenated sequence of L. kimchii KCTC 2386T (94.2%). Lower concatenated sequence similarities (<88.1%) were found towards other species of the genus Leuconostoc with validly published names. The pheS, rpoA and atpA gene sequences of strain LMG 27676T revealed high similarities to the sequences of L. kimchii KCTC 2386T (91.3, 96.4 and 94.4% similarity, respectively). Lower gene sequence similarities were found (<84.5, 89 and 91.5%, respectively) towards other species of the genus Leuconostoc with validly published names. According to De Bruyne et al. (2007), species now classified in the genera Leuconostoc and Fructobacillus are delineated above 93, 98 and 98% pheS, rpoA and atpA gene sequence similarity, respectively. Therefore, these results suggest that strain LMG 27676T represents a novel species of the genus Leuconostoc. To confirm this, a DNA–DNA hybridization experiment between strain LMG 27676T and L. kimchii strains KCTC 2386T and LMG 23786 was performed. Genomic DNA was extracted using the guanidine thiocyanate method described by Pitcher et al. (1989). The DNA–DNA hybridization experiment was performed using the microplate method, with photobiom for labelling of the DNA (Ezaki et al., 1989), as modified by Goris et al. (1998). The hybridization level of strain LMG 27676T towards L. kimchii KCTC 2386T was 51% (the reciprocal hybridization values were 53 and 49%); the hybridization level between L. kimchii KCTC 2386T and L. kimchii LMG 23786 was 85% (the reciprocal hybridization values were 72 and 97%); and the hybridization level between strain LMG 27676T and L. kimchii LMG 23786 was 64% (the reciprocal hybridization values were 70 and 57%).

The hybridization values between strain LMG 27676T and both L. kimchii strains were clearly below the species delineation threshold.

DNA G+C content was determined according to the enzymic DNA degradation method described by Mesbah & Whitman (1989), using a Waters Breeze HPLC system and XBridge Shield RP18 column. The solvent used was 0.02 M NH₄H₂PO₄ (pH 4.0)/1.5% (v/v) acetonitrile. Non-methylated lambda phage DNA (Sigma-Aldrich) was used as a calibration reference and Escherichia coli LMG 2093 DNA was included as a control. The DNA G+C content of strain LMG 27676T was 38 mol%, which is consistent with the DNA G+C contents found previously in members of the genus Leuconostoc (38–44 mol%; De Bruyne et al., 2007).

Preparation of peptidoglycan and analysis of the peptido-glycan structure were performed according to published protocols (Schumann, 2011). The total hydrolysate (4 M HCl, 100 °C, 16 h) contained the amino acids alanine, glutamic acid and lysine, and analysis of partial hydrolysates (4 M HCl, 100 °C, 45 min) revealed the presence of the
peptidoglycan type A3α l-Lys-Ala2 or type A11.5 according to www.peptidoglycan-types.info.

Finally, a biochemical analysis of LMG 27676T, KCTC 2386T and LMG 23786 was performed to characterize the novel species. Cell and colony morphology of strain LMG 27676T was verified after growth on MRS agar (Oxoid) and 96 h of aerobic incubation at 28 °C. Conventional biochemical characteristics and enzyme activities were tested as described previously in triplicate, unless stated otherwise (De Bruyne et al., 2007). Growth was tested at 4, 20, 30, 37 and 45 °C and in the presence of 0, 2, 4, 6, 8 and 10 % NaCl (w/v). The production of gas from glucose was determined using MRS broth without tri-ammonium citrate and inverted Durham tubes, and carbohydrate fermentation profiles were determined using the API 50 CHL Lactobacillus identification system (bioMérieux). Arginine hydrolysis was evaluated in a medium containing 0.5 % tryptone, 0.5 % yeast extract, 0.3 % l-arginine, 0.05 % glucose, 0.2 % K2HPO4 (pH 7.0), with methyl orange as an indicator. To test for the production of D- and L-lactate from glucose, cells were grown in MRS broth with glucose as the sole energy source. Proteins were removed by adding an equal volume of acetonitrile (Sigma-Aldrich); samples were subsequently microcentrifuged (10 000 g for 8 min), filtered (0.2 μm; Sartorius AG) and transferred to an appropriate vessel prior to injection. The amount of D- and L-lactic acid in the supernatant was determined using HPLC with UV detection. To this end, a Waters chromatograph was used, equipped with a 486 UV detector, a 600S controller, a 717Plus autosampler and a Spherisorb-Column Orpak CRX-853 (50 x 8 mm; Showa Denko KK). The mobile phase, at a flow rate of 1 ml min⁻¹, consisted of a 1 mM CuSO4 solution in ultrapure water with 20 % acetonitrile (Sigma-Aldrich). D- and L-lactic acids were eluted isocratically and were detected by measuring the absorption at 253 nm. The results for strain LMG 27676T are given in the species description below. Characteristics that differentiate strain LMG 27676T from its nearest phylogenetic neighbours are summarized in Table 1. The biochemical characteristics of KCTC 2386T and LMG 23786 were identical with the following exceptions: only KCTC 2386T produces acid from l-arabinose, whereas only LMG 23786 produces acid from arbutin.

In conclusion, the results of the present study demonstrate that strain LMG 27676T represents a taxon that can be differentiated from the present species of the genus *Leuconostoc* by a range of genotypic methods and by several biochemical characteristics including the absence of growth at 37 °C. We therefore classify the taxon represented by strain LMG 27676T as a novel species of the genus *Leuconostoc*, for which we propose the name *Leuconostoc rapi* sp. nov.

**Description of *Leuconostoc rapi* sp. nov**

*Leuconostoc rapi* (ra’pi. L. gen. n. rapi of a turnip, refering to rutabaga, a turnip-like vegetable).

Cells are Gram-stain-positive, catalase-negative, facultatively anaerobic and non-motile. Cells are ovoid and occur singly, in pairs or in short chains. Colonies grown for 3 days on MRS agar at 28 °C are approximately 1 mm in diameter, white/creamy, convex and circular with smooth margins. Gas is produced from glucose, indicating the heterofermentative character of the type strain. Production of l- and d-lactic acid in a ratio of 2 : 7. Growth occurs at 20–30 °C and in the presence of 0–6% NaCl but not in the presence of 8–10% NaCl. Arginine is not hydrolysed. Acid is produced from glucose, fructose, mannose, l-arabinose, d-ribose, mannitol, methyl α-D-glucopyranoside, N-acetylglucosamine, amygdalin, aesculin.

### Table 1. Differential phenotypic characteristics of strain LMG 27676T and members of the closest related species of the genus *Leuconostoc*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Rutabaga</td>
<td>Kimchi</td>
<td>Brown algae kimchi</td>
</tr>
<tr>
<td>Morphology</td>
<td>Ovoid</td>
<td>Coccus</td>
<td>Ovoid</td>
</tr>
<tr>
<td>NaCl (%) range for growth</td>
<td>0–6</td>
<td>0–7</td>
<td>0–6</td>
</tr>
<tr>
<td>Growth at 4 °C</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 37 °C</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acid production (API 50 CHL) from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-Arabinose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Arbutin</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>d-Xylose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>38</td>
<td>37</td>
<td>42.5</td>
</tr>
</tbody>
</table>

*This biochemical characteristic differs from what was previously reported (Kim et al., 2000).*
ferric citrate, salicylic, cellobiose, sucrose, trehalose, gen-
tiobiose, turanose, potassium gluconate and potassium
2-ketogluconate. Acid is not produced from glycerol, ery-
thritol, D-arabinose, D- and L-xyllose, D-adenitol, methyβ-
D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose,
dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside,
arbutin, maltose, lactose, melibiose, inulin, melezitose, raffi-
nose, starch, glycerogen, xylitol, D-lyxose, D-tagatose, D- or L-
fucrose or D- or L-arabitol.

The type strain, LMG 27676^T (= R-50029^T=MHB 277^T=
DSM 27776^T), was isolated from a spoiled sous-vide-
cooked rutabaga produced by a Finnish manufacturer.
The DNA G+C content of the type strain is 38 mol% and its peptidoglycan type is A3γ L-Lys-Ala-2α.

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