Leuconostoc mesenteroides subsp. suionicum subsp. nov.

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Strains LMG 8159 and LMG 11499 were reclassified by a polyphasic approach, including 16S rRNA gene sequence analysis, 16S–23S rRNA intergenic spacer (IGS) sequence analysis, (GTG)₅-PCR fingerprinting, RAPD fingerprinting, fatty acid methyl ester analysis and an analysis of phenotypic features using API 50 CH. The two strains were closely related to the type strains of the three defined subspecies of Leuconostoc mesenteroides, showing 99.7–99.9 % 16S rRNA gene sequence similarity, 99.2 % 16S–23S rRNA gene intergenic spacer sequence similarity, 97.1–97.4 % pheS gene sequence similarity and 98.0–98.2 % rpoA gene sequence similarity. Low atpA gene sequence similarity (91.4–91.7 %), (GTG)₅-PCR fingerprinting, RAPD fingerprinting, fatty acid compositions and phenotypic features allowed us to differentiate strains LMG 8159 and LMG 11499 from all established subspecies within L. mesenteroides. Based upon the data obtained in the present and previous studies, a novel subspecies is proposed within the species L. mesenteroides, Leuconostoc mesenteroides subsp. suionicum subsp. nov., with the type strain LMG 8159T (=ATCC 9135T =DSM 20241T =NCIMB 6992T).

Abbreviations: FAME, fatty acid methyl ester; IGS, intergenic spacer; RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 11499 and LMG 8159 and L. pseudomesenteroides LMG 11482T are HM443065, HM443097 and HM443095, and those for the 16S–23S rRNA IGS sequences of L. mesenteroides subsp. dextranicum LMG 60908T, L. mesenteroides subsp. cremoris LMG 6909T and strains LMG 8159 and LMG 11499 are HM443064–HM443066 and HM443069.

Four supplementary figures and two supplementary tables are available with the online version of this paper.

Leuconostocs are usually found in plant material, milk, dairy products, meat and other food products. After a series of taxonomic changes since the 1980s, the genus Leuconostoc contains 12 recognized species, including two species described relatively recently, Leuconostoc holzapffeli (de Bruyne et al., 2007) and Leuconostoc palmiae (Ehrmann et al., 2009). Currently, the type species Leuconostoc mesenteroides consists of three subspecies: L. mesenteroides subsp. mesenteroides, L. mesenteroides subsp. dextranicum and L. mesenteroides subsp. cremoris (Garvie, 1983).

In previous studies (Garvie, 1976; Hontebeyrie & Gasser, 1977; Farrow et al., 1989), strain LMG 8159 (formerly NCDO 797, NCDO 522, NCIB 9317 and NRRL B-523) showed 79–91 % DNA–DNA hybridization with L. mesenteroides subsp. mesenteroides DSM 5893T, confirming that the strain belongs to L. mesenteroides. In a more recent study (de Bruyne et al., 2007), strains LMG 8159 and LMG 11499 formed a distinct clade and were related to L. mesenteroides in a concatenated phylogenetic tree based upon pheS, rpoA and atpA gene sequences. Gene sequence similarities between strain LMG 8159 and the type strains of phylogenetically related species are shown in Table 1. Strains LMG 8159 and LMG 11499 had almost identical pheS, rpoA and atpA gene sequences (99.8–100 % similarity), and they were closely related to L. mesenteroides based upon pheS and rpoA sequence analyses. In contrast, low atpA gene sequence similarity (91.4–92.7 %) was found between strains LMG 8159 and LMG 11499 and the type strains of L. mesenteroides subsp. mesenteroides, L. mesenteroides subsp. dextranicum and L. mesenteroides subsp. cremoris, suggesting that strains LMG 8159 and LMG 11499 represent a novel subspecies within the species L. mesenteroides.

In the present study, strains LMG 8159 and LMG 11499 were studied further in order to clarify whether they could be assigned to a novel subspecies within the species L.
mesenteroides. The strains used in this study are listed in Table 2. All strains were incubated aerobically at 30 °C on MRS medium.

16S rRNA gene and 16S–23S rRNA intergenic spacer (IGS) sequence analyses were performed to investigate further the phylogenetic relationships of strains LMG 8159 and LMG 11499. Amplification of the 16S rRNA gene was performed using the primers of An et al. (2006). The 16S–23S rRNA IGS was amplified using the primers and protocol of Rachman et al. (2003). Purification and sequencing of PCR products were carried out by the Shenggong Company (Shanghai, China). The resulting sequences, together with those of related strains obtained from the GenBank database, were aligned by CLUSTAL W. A phylogenetic tree was constructed using the neighbour-joining method with the maximum composite likelihood model. Bootstrap analysis was performed based on 1000 replicates. The MEGA4 package (Tamura et al., 2007) was used for all analyses. Strains LMG 8159 and LMG 11499 were highly related, having 99.9 % 16S rRNA gene sequence similarity (Table 1; Fig. 1) and 99.2 % 16S–23S rRNA IGS sequence similarity (Fig. S1, available in IJSEM Online). 16S rRNA gene sequence similarity between strain LMG 8159 and the type strains of phylogenetically related species was at least 99.5 %: similarities of 99.9, 99.9, 99.7 and 99.5 % were shown to strains LMG 8159 and LMG 11499 are shown in Table S1, and more features are presented in the subspecies description. Strains LMG 8159 and LMG 11499 showed 96 % similarity, and differed from the type strains of L. mesenteroides subsp. mesenteroides, L. mesenteroides subsp. cremoris and L. pseudomesenteroides at a similarity level of 92 %. Distinctive features of strains LMG 8159 and LMG 11499 are shown in Table S1, and more features are presented in the subspecies description. Strains LMG 8159 and LMG 11499 are easily distinguished from L. mesenteroides subsp. dextranicum and L. mesenteroides subsp. cremoris. These two strains can also be differentiated from L. mesenteroides subsp. mesenteroides because they did not ferment raffinose.

Whole-cell fatty acids were analysed as fatty acid methyl esters (FAMEs) using the MIDI Microbial Identification system with database TSBA6. Cultures were incubated for 3 days at 30 °C on MRS solid medium. FAMEs were extracted and prepared according to the protocol of Sasser (1990). Fatty acid compositions of strains LMG 8159 and LMG 11499 and the type strains of L. mesenteroides subsp.

### Table 1. Sequence similarities (%) between strain LMG 8159 and phylogenetically related reference strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>16S rRNA</th>
<th>pheS</th>
<th>rpoA</th>
<th>atpA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMG 11499</td>
<td>99.9</td>
<td>100</td>
<td>99.8</td>
<td>99.9 (100)</td>
</tr>
<tr>
<td>L. mesenteroides subsp. mesenteroides LMG 6893&lt;sup&gt;T&lt;/sup&gt;</td>
<td>99.9</td>
<td>97.4</td>
<td>98.2</td>
<td>91.5 (92.4)</td>
</tr>
<tr>
<td>L. mesenteroides subsp. dextranicum LMG 6908&lt;sup&gt;T&lt;/sup&gt;</td>
<td>99.9</td>
<td>97.1</td>
<td>98.0</td>
<td>91.7 (92.7)</td>
</tr>
<tr>
<td>L. mesenteroides subsp. cremoris LMG 6909&lt;sup&gt;T&lt;/sup&gt;</td>
<td>99.7</td>
<td>97.4</td>
<td>98.0</td>
<td>91.4 (92.3)</td>
</tr>
<tr>
<td>L. pseudomesenteroides LMG 11482&lt;sup&gt;T&lt;/sup&gt;</td>
<td>99.5</td>
<td>73.9</td>
<td>84.7</td>
<td>88.4 (89.3)</td>
</tr>
</tbody>
</table>

IGS sequence analysis clearly showed greater resolution than 16S rRNA gene sequence analysis, again confirming that strains LMG 8159 and LMG 11499 belong to L. mesenteroides.

The API 50 CH system (bioMérieux) was used to analyse phenotypic features that differentiated strains LMG 8159 and LMG 11499 from related species. Test preparations were incubated at 30 °C, and readings were made after 48 h. In a cluster analysis (Fig. S2) using the $S_m$ coefficient ($S_m = \Sigma I / 49$, where $\Sigma I$ is the number of features identical for the compared strain pair and 49 is the number of total tested features) and the unweighted pair-group method with arithmetic means (UPGMA) (Sneath & Sokal, 1973), strains LMG 8159 and LMG 11499 showed 96 % similarity, and differed from the type strains of L. mesenteroides subsp. mesenteroides, L. mesenteroides subsp. dextranicum, L. mesenteroides subsp. cremoris and L. pseudomesenteroides at a similarity level of 92 %. Distinctive features of strains LMG 8159 and LMG 11499 are shown in Table S1, and more features are presented in the subspecies description. Strains LMG 8159 and LMG 11499 are easily distinguished from L. mesenteroides subsp. dextranicum and L. mesenteroides subsp. cremoris. These two strains can also be differentiated from L. mesenteroides subsp. mesenteroides because they did not ferment raffinose.

### Table 2. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. mesenteroides subsp. suionicum subsp. nov.</td>
<td>1972, Sweden. Identified as L. mesenteroides subsp. mesenteroides</td>
</tr>
<tr>
<td>LMG 8159&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Not known. Identified as L. pseudomesenteroides</td>
</tr>
<tr>
<td>LMG 11499</td>
<td>Fermenting olives; 1941, USA</td>
</tr>
<tr>
<td>L. mesenteroides subsp. mesenteroides LMG 6893&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Isolated in 1912</td>
</tr>
<tr>
<td>L. mesenteroides subsp. dextranicum LMG 6908&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Hansen’s dried cheese starter powder</td>
</tr>
<tr>
<td>L. mesenteroides subsp. cremoris LMG 6909&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Cane juice</td>
</tr>
<tr>
<td>L. pseudomesenteroides LMG 11482&lt;sup&gt;T&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>
mesenteroides, L. mesenteroides subsp. dextranicum and L. mesenteroides subsp. cremoris are given in Table S2.

Repetitive element palindromic-PCR with the primer (GTG)5 and randomly amplified polymorphic DNA (RAPD) analysis were performed in order to investigate genetic relationships among strains LMG 8159 and LMG 11499 and phylogenetically related species. (GTG)5-PCR was performed according to the method of Švec et al. (2008). RAPD amplifications using five different primers, 1254, 1281, D8635, D14216 and M13, were carried out separately as described previously by Torriani et al. (1999) and Akopyanz et al. (1992). PCR products were separated by electrophoresis in 2 % agarose gels. Strains LMG 8159 and LMG 11499 had similar patterns, which were distinguishable from those of L. mesenteroides subsp. mesenteroides LMG 6893T, L. mesenteroides subsp. dextranicum LMG 6908T, L. mesenteroides subsp. cremoris LMG 6909T and L. pseudomesenteroides LMG 11482T (Figs S3 and S4).

In conclusion, strains LMG 8159 and LMG 11499 were highly similar based upon the present data and previous observations (de Bruyne et al., 2007), and they were classified as L. mesenteroides by 16S rRNA gene sequence analysis (Fig. 1), 16S–23S rRNA IGS sequence analysis (Fig. S1), phoS and rpoA sequence analysis (Table 1) and DNA–DNA hybridization (Garvie, 1976; Hontebeyrie & Gasser, 1977; Farrow et al., 1989). However, strains LMG 8159 and LMG 11499 could be differentiated from the three defined subspecies of L. mesenteroides by atpA sequence analysis (Table 1), (GTG)5-PCR fingerprinting (Fig. S3), RAPD fingerprinting (Fig. S4), analysis of phenotypic features using API 50 CH (Table S1) and FAME analysis (Table S2). On the basis of the data obtained in the present and previous studies (Garvie, 1976; Hontebeyrie & Gasser, 1977; Farrow et al., 1989; de Bruyne et al., 2007), a novel subspecies within the species L. mesenteroides is proposed, with the name Leuconostoc mesenteroides subsp. suionicum subsp. nov.

The characteristics that differentiate L. mesenteroides subsp. suionicum subsp. nov. from the three recognized subspecies of L. mesenteroides include low atpA gene sequence similarities (<92 %), (GTG)5-PCR fingerprinting, RAPD fingerprinting, fatty acid compositions and phenotypic features. Strains of L. mesenteroides subsp. suionicum do not ferment raffinose, whereas strains of L. mesenteroides subsp. mesenteroides do. Strains of L. mesenteroides subsp. suionicum produce acid from L-arabinose, aesculin, salicin and cellobiose, whereas strains of L. mesenteroides subsp. dextranicum give the opposite reactions. Strains of L. mesenteroides subsp. cremoris do not ferment L-arabinose, D-xylene, aesculin, salicin, cellobiose, maltose or melibiose, whereas strains of L. mesenteroides subsp. suionicum give the opposite reactions.

The content of FAMES C16:0 and C19:0 cyclo 9c and C16:0 can distinguish L. mesenteroides subsp. suionicum from L. mesenteroides subsp. dextranicum and L. mesenteroides subsp. cremoris. The content of FAMES C18:1o9c and C18:1o6c/C18:1o7c can differentiate L. mesenteroides subsp. suionicum from L. mesenteroides subsp. mesenteroides and L. mesenteroides subsp. cremoris.

**Description of Leuconostoc mesenteroides subsp. suionicum subsp. nov.**

*Leuconostoc mesenteroides subsp. suionicum* [su.i.o’ni.cum]. L. n. *Suioni* according to Tacitus, the people who live in Scandinavia; L. neut. suffix -icum belonging to; N.L. neut. adj. suionicum belonging to (or coming from) Sweden.

Gram-stain-positive. Non-spore-forming coccus. Catalase is not produced. Facultatively anaerobic. Optimum growth temperature is 30 °C; can grow at 37 °C. Acid is produced from L-arabinose, ribose, D-xylene, galactose, glucose, fructose, mannose, methyl α-D-glucopyranoside, N-acetylgalactosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, melibiose, sucrose, trehalose, gentiobiose and turanose. Acid is not produced from glycerol, erythritol, D-arabinose, L-xylose, adonitol, methyl β-D-xylopyranoside, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-mannopyranoside, inulin, melezitose, raffinose, starch, glycolen, xylitol, lyxose, tagatose, D- or L-fucose, D- or L-arabinitol, gluconate or 2- or 5-ketogluconate.
The type strain is strain LMG 8159\textsuperscript{T} (=ATCC 9135\textsuperscript{T} =DSM 20241\textsuperscript{T} =NCIMB 6992\textsuperscript{T}). The type strain can produce acid from mannitol and lactose. The DNA G+C content of the type strain is 36.4 mol\% (Farrow et al., 1989). Strain LMG 11499 is a second member of the subspecies.

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References


