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 8159^T (=ATCC 9135^T =DSM 20241^T =NCIMB 6992^T).

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 holzapfelii* (de Bruyne *et al.*, 2007) and *Leuconostoc palmae* (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* LMG 6893^T, 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.*

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 11482^T are HM443955, HM443957 and HM443958, and those for the 16S–23S rRNA IGS sequences of *L. mesenteroides* subsp. *dextranicum* LMG 6908^T, *L. mesenteroides* subsp. *cremoris* LMG 6909^T and strains LMG 8159 and LMG 11499 are HM443964–HM443966 and HM443969.

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

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

Data were obtained in this study or taken from de Bruyne *et al.* (2007). Values in parentheses refer to sequence similarities with strain LMG 11499.

Strain	16S rRNA	<i>pheS</i>	<i>rpoA</i>	<i>atpA</i>
LMG 11499	99.9	100	99.8	99.9 (100)
<i>L. mesenteroides</i> subsp. <i>mesenteroides</i> LMG 6893 ^T	99.9	97.4	98.2	91.5 (92.4)
<i>L. mesenteroides</i> subsp. <i>dextranicum</i> LMG 6908 ^T	99.9	97.1	98.0	91.7 (92.7)
<i>L. mesenteroides</i> subsp. <i>cremoris</i> LMG 6909 ^T	99.7	97.4	98.0	91.4 (92.3)
<i>L. pseudomesenteroides</i> LMG 11482 ^T	99.5	73.9	84.7	88.4 (89.3)

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 the type strains of *L. mesenteroides* subsp. *mesenteroides*, *L. mesenteroides* subsp. *dextranicum*, *L. mesenteroides* subsp. *cremoris* and *Leuconostoc pseudomesenteroides*, respectively (Table 1). 16S–23S rRNA IGS sequence similarities between strain LMG 8159 and the type strains of *L. mesenteroides* subsp. *mesenteroides*, *L. mesenteroides* subsp. *dextranicum*, *L. mesenteroides* subsp. *cremoris* and *L. pseudomesenteroides* were 99.2, 99.2, 99.2 and 95.0 %, respectively. 16S–23S rRNA

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 ΣI 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.

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 2. Bacterial strains used in this study

Strain	Source
<i>L. mesenteroides</i> subsp. <i>suionicum</i> subsp. nov. LMG 8159 ^T LMG 11499	1972, Sweden. Identified as <i>L. mesenteroides</i> subsp. <i>mesenteroides</i> Not known. Identified as <i>L. pseudomesenteroides</i>
<i>L. mesenteroides</i> subsp. <i>mesenteroides</i> LMG 6893 ^T	Fermenting olives; 1941, USA
<i>L. mesenteroides</i> subsp. <i>dextranicum</i> LMG 6908 ^T	Isolated in 1912
<i>L. mesenteroides</i> subsp. <i>cremoris</i> LMG 6909 ^T	Hansen's dried cheese starter powder
<i>L. pseudomesenteroides</i> LMG 11482 ^T	Cane juice

Fig. 1. Neighbour-joining tree showing the phylogenetic relationships of the test strains and reference strains based on 16S rRNA gene sequences. Bar, 0.002 substitutions per site.

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 6893^T, *L. mesenteroides* subsp. *dextranicum* LMG 6908^T, *L. mesenteroides* subsp. *cremoris* LMG 6909^T and *L. pseudomesenteroides* LMG 11482^T (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), *pheS* 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-xylose, aesculin, salicin, cellobiose, maltose or melibiose, whereas strains of *L. mesenteroides* subsp. *suionicum* give the opposite reactions. The content of FAMES C_{16:0} and C_{19:0} cyclo ω8c can distinguish *L. mesenteroides* subsp. *suionicum* from *L. mesenteroides* subsp. *dextranicum* and *L. mesenteroides* subsp. *cremoris*. The content of FAMES C_{18:1}ω9c and C_{18:1}ω6c/C_{18:1}ω7c 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-xylose, galactose, glucose, fructose, mannose, methyl α-D-glucopyranoside, N-acetylglucosamine, 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, glycogen, xylitol, lyxose, tagatose, D- or L-fucose, D- or L-arabitol, gluconate or 2- or 5-ketogluconate.

The type strain is strain LMG 8159^T (=ATCC 9135^T =DSM 20241^T =NCIMB 6992^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.

Acknowledgements

This work was supported by the Program for Changjiang Scholars and Innovative Research Team in University (IRT0959), the Science Foundation of North-east Agricultural University of China, and the National Natural Science Foundation of China (31101342).

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