Reclassification of *Leuconostoc gasicomitatum* as *Leuconostoc gelidum* subsp. *gasicomitatum* comb. nov., description of *Leuconostoc gelidum* subsp. *aenigmaticum* subsp. nov., designation of *Leuconostoc gelidum* subsp. *gelidum* subsp. nov. and emended description of *Leuconostoc gelidum*

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In the present study we investigated the taxonomic status of 20 lactic acid bacteria (LAB) originating from packaged meat. On the basis of 16S rRNA gene sequence similarity, these strains were shown to belong to the genus *Leuconostoc* with *Leuconostoc gelidum*, *Leuconostoc inhae* and *Leuconostoc gasicomitatum* as the closest phylogenetic relatives. The novel strains shared more than 70% DNA–DNA relatedness with type and reference strains of both *L. gelidum* and *L. gasicomitatum*. The DNA–DNA relatedness values between *L. gelidum* type and reference strains and *L. gasicomitatum* type and reference strains were also above 70%, showing that all these strains belonged to the same species. Sequence analyses of concatenated *atpA*, *pheS*, and *rpoA* genes demonstrated that the novel strains as well as type and reference strains of *L. gelidum* and *L. gasicomitatum* are phylogenetically closely related, but form three clearly separated subgroups. Numerical analysis of HindIII ribopatterns and phenotypic tests supported this subdivision. Based on the data presented in this study, we propose to reclassify *Leuconostoc gasicomitatum* as *Leuconostoc gelidum* subsp. *gasicomitatum* comb. nov. (type strain, LMG 18811T = DSM 15947T). The novel strains isolated in the present study represent a novel subspecies, for which the name *Leuconostoc gelidum* subsp. *aenigmaticum* subsp. nov. is proposed, with POUF4dT ( =LMG 27840T =DSM 19375T) as the type strain. The proposal of these two novel subspecies automatically creates the subspecies *Leuconostoc gelidum* subsp. *gelidum* subsp. nov. (type strain, NCFB 2775T =DSM 5578T). An emended description of *Leuconostoc gelidum* is also provided.

During characterization of spoilage microbiota of packaged meat, we isolated 20 lactic acid bacteria (LAB) strains from vacuum-packaged pork, vacuum-packaged turkey and modified atmosphere-packaged (MAP) broiler that remained unidentified in the numerical analysis of HindIII ribopatterns (Fig. S1, available in the online Supplementary Material). The 20 LAB strains formed a tight cluster that was close to but clearly separated from *Leuconostoc gelidum* and *Leuconostoc gasicomitatum*. *L. gasicomitatum* and *L. gelidum* are closely related and form a phylogenetic branch within the genus *Leuconostoc* together with *Leuconostoc inhae*, *Leuconostoc kimchii*, *Leuconostoc carnosum* and the recently described species *Leuconostoc miyukkimchii* (Björkroth & Holzapfel, 2006; Lee et al., 2012). Characteristic for these species of the genus *Leuconostoc*, with the

**Abbreviations:** DDH, DNA–DNA hybridization; LAB, lactic acid bacteria; MAP, modified atmosphere-packaged; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene, *atpA*, *pheS*, and *rpoA* sequences of *Leuconostoc gelidum* subsp. *aenigmaticum* POUF4dT are KF577569, KF577549, KF577560 and KF577564, respectively. The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene, *atpA* and *pheS* gene sequences of the reference strains of *Leuconostoc gelidum* subsp. *aenigmaticum* are KF577567–KF577568, KF577560–KF577564 and KF577555–KF577559, respectively, and those for the *rpoA* gene sequences are KF577561–KF577563 and KF577565–KF577566 (see Fig. 1 and Table S1 for details).

Two supplementary tables and three supplementary figures are available with the online version of this paper.

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10.1099/ijs.0.058263-0
exception of L. kimchii, is the ability to grow at low temperatures, which enables them to grow in chill-stored food (Björkroth & Holzapfel, 2006). L. gelidum and L. gasicomitatum often form part of the dominant microbiota in late shelf-life, packaged meat, and have been associated with spoilage of a variety of meat and meat products (Björkroth & Holzapfel, 2006).

The purpose of the present study was to resolve the taxonomic status of the novel strains by a polyphasic approach including phenotypic tests, sequence analysis of 16S rRNA, atpA, pheS and rpoA genes, and DNA–DNA hybridization. In this study we show that the strains represent a novel subspecies of the species L. gelidum. We also propose to reclassify the species L. gasicomitatum as a subspecies of L. gelidum.

The 20 strains were isolated from MAP meat by growth on Man-Rogosa-Sharpe agar (MRS; Oxoid) at 25 °C for 5 days under anaerobic conditions [Anaerogen (Oxoid); 9–13 % CO2 according to the manufacturer]. All isolates were maintained in MRS broth at 2°C and grown in MRS broth and agar at 25 °C in anaerobic jars. The sources of the isolates of the genus Leuconostoc are presented in Fig. S1.

The nearly complete 16S rRNA gene sequence of three representative unknown strains was determined to confirm the genus level identification of the isolates as described by Vihavainen & Björkroth (2007). DNA for all analyses was extracted as described by Pitcher et al. (1989) with modifications as described by Björkroth & Korkeala (1996). Sequences of the isolates were subjected to the BLAST search program (Altschul et al., 1997), the 16S rRNA sequences of the type strains of species within the genus Leuconostoc were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov), and all the sequences were aligned with CLUSTAL_X (Thompson et al., 1994). A phylogenetic tree was reconstructed by PALM (Chen et al., 2009) by using the maximum-likelihood method with bootstrap values based on 1000 replications.

Sequence analysis of the 16S rRNA gene clearly showed that the isolates belonged to the genus Leuconostoc (Fig. 1). The 16S rRNA gene sequences of the unknown strains POUF4dT, AMKR32 and POKY4-4 were identical, and most similar to L. gelidum DSM 5578T, L. gasicomitatum LMG 18811T, and L. inhae KCTC 3774T (99.8, 99.7 and 99.2 % sequence similarity, respectively). The similarity between L. gelidum DSM 5578T and L. gasicomitatum LMG 18811T was 99.4 %. The high 16S rRNA gene sequence similarity within the L. gelidum lineage has been reported before (Björkroth et al., 2000).

Multilocus sequence analysis (MLSA) of housekeeping genes atpA, pheS and rpoA has been successfully applied for

**Fig. 1.** Maximum-likelihood tree based on 16S rRNA gene sequences of three representative novel strains and 13 type and reference strains of species of the genus Leuconostoc, with Leuconostoc fallax LMG 13177T as an outgroup. GenBank accession numbers are given in parentheses. Bootstrap values >50 % after 1000 resamplings are shown at branching points. Bar, 0.1 substitutions per nucleotide position.
differentiation of species of the genus *Leuconostoc*, and the discriminatory resolution of these genes was shown to be substantially higher compared to the sequence of the 16S rRNA gene (De Bruyne et al., 2007). Sequences of the *atpA*, *pheS* and *rpoA* genes of the unknown strains were determined to investigate the taxonomic position of the isolates. Amplification and sequencing was performed as described by Naser et al. (2005a, b). The sequences were assembled using the BioNumerics software package (Applied Maths), subjected to the BLAST search program (Altschul et al., 1997), and aligned with sequences of type strains of species of the genus *Leuconostoc* retrieved from the GenBank database using CLUSTAL_X software (Thompson et al., 1994). GenBank/EMBL accession numbers for the *atpA*, *pheS* and *rpoA* genes used in this study are given in Table S1. Phylogenetic trees were reconstructed using the maximum-likelihood method (Fig. 2) as described above for 16S rRNA gene sequence analysis and by CLUSTAL_X using the neighbour-joining method (Fig. S2).

In the maximum-likelihood tree based on concatenated sequences of *atpA*, *pheS* and *rpoA* genes, the six representative unknown strains formed a distinct branch close to two clusters formed by the four strains of *L. gasicomitatum* and the three strains of *L. gelidum* (Fig. 2). The topology of the trees reconstructed using maximum-likelihood and neighbour-joining methods are mostly congruent except for the position of *L. citreum* (with low bootstrap values in both trees) and the relative positions of the novel strains, *L. gasicomitatum* and *L. gelidum* in spite of their clustering in the same clade (Fig. S2). In the analysis of the partial *pheS* sequences (Fig. S3), which is the most discriminatory locus, the similarity between strains POUF4dT and AMKR32 was 98.7 %, and the similarity between strains POUF4dT and both *L. gasicomitatum* LMG 18811T and *L. gelidum* LMG 18297T was 96.1 %. Between *L. gasicomitatum* LMG 18811T and *L. gelidum* LMG 18297T, the *pheS* sequence similarity was 93.2 %, and for *L. inhae* LMG 22919T, the similarity with strain POUF4dT, *L. gasicomitatum* LMG 18811T and *L. gelidum* LMG 18297T, was 89.6, 89.9 and 90.6 %, respectively. This correlates with the study by De Bruyne et al. (2007), who reported that species of the genus *Leuconostoc* were delineated above 93 % *pheS* sequence similarity.

For DNA–DNA hybridization (DDH), DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DDH was carried out as described by De Ley et al. (1970) under consideration of

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**Fig. 2.** Maximum-likelihood tree based on concatenated *atpA*, *pheS* and *rpoA* sequences of *Leuconostoc gelidum* subsp. *aenigmaticum* subsp. nov. and 16 type and reference strains of species of the genus *Leuconostoc* with *Leuconostoc fallax* LMG 13177T as an outgroup. Bootstrap values >50 % after 1000 resamplings are shown at branching points. Bar, 0.07 substitutions per site.
the modifications described by Huss et al. (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with in situ temperature probe (Varian). The DDH experiment was repeated in another laboratory with the same spectrophotometric method of De Ley et al. (1970) as described by De Bruyne et al. (2007). The DNA base composition analysis of strain POUF4dT was performed as described by De Bruyne et al. (2007).

The DDH values between strain POUF4dT and L. gasi- comitatum LMG 18811T, L. gelidum DSM 5578T, and L. inhae LMG 22919T were 75 ± 2, 76 ± 3 and 45 ± 4 %, respectively. This shows that strain POUF4dT belongs to the same species as L. gasicomitatum LMG 18811T and L. gelidum DSM 5578T, and does not belong to the same species as L. inhae LMG 22919T when the cut-off point of 70 % DNA–DNA relatedness for species delineation is considered (Wayne et al., 1987). Strains POUF4dT and AMKR32 showed a DDH value of 87 ± 4 %. The DDH values for L. gasicomitatum LMG 18811T with L. gasicomitatum LMG 19597, L. gelidum DSM 5578T and L. gelidum AMKR21 were 82 ± 7, 80 ± 4, and 80 ± 1 %, respectively, indicating that these strains belong to the same species. The DDH values for L. gasicomitatum LMG 18811T with L. inhae LMG 22919T and L. mesenteroides DSM 20343T were 51 ± 3 and 35 ± 2 %, respectively. Thus, these strains do not represent the same species. The DNA–DNA relatedness values obtained for L. gasicomitatum LMG 18811T and L. gelidum DSM 5578T were clearly higher than those reported by Björkroth et al. (2000) and Kim et al. (2000), who assigned these strains to different species. We thus repeated the DDH experiment in another laboratory, which confirmed our results (Table S2). The DNA G + C content of strain POUF4dT was 37 mol%, which is within the expected range of the genus Leuconostoc.

Phenotypic characteristics were tested for six representa- tive strains (AMKR32, APM2b4, POKY4-4, POKY4-10, POUF4dT and POUF4h). Growth at 0, 5, 10, 15, 25, 30 and 37 °C, pH 2–10 and with 2, 4, 6.5, and 8 % NaCl was tested in MRS broth (Oxoid) until growth was observed or for at least 21 days. The carbohydrate fermentation profiles of the strains were studied using API 50 CHL and API Strep (bioMérieux) according to the manufacturer’s instructions. Tests for production of ammonia from arginine, gas from glucose and slime from sucrose were performed as described by Koort et al. (2004). Like other Leuconostocs, cells were Gram-positive, catalase-negative, facultatively anaerobic, oval cocci. The API 50CH profiles of the novel strains were similar to the profile of the type strain of L. gasicomitatum (Table 1). Like L. gasicomitatum, and unlike L. gelidum, the novel strains did not ferment amygdalin, arbutin or salicin. All strains produced gas from glucose, and none of the strains produced ammonium from arginine. The strains grew at 5 °C and at 25 °C, but not at 37 °C; two out of six strains grew at 30 °C. The strains grew in 4 % NaCl, and at pH 5–8. Unlike L. gasicomitatum, five out of six strains, including the proposed type strain POUF4dT, grew in 6.5 % NaCl.

### Table 1. Phenotypic characteristics of Leuconostoc gelidum subsp. aenigmaticum subsp. nov. and its closest phylogenetic relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tr>
<td>Growth in 6.5 % NaCl</td>
<td>(83)</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>ND</td>
<td>+</td>
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<tr>
<td>Haem-stimulated aerobic growth</td>
<td>−</td>
<td>+</td>
<td>*</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Acid production from:</td>
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<tr>
<td>Amygdalin</td>
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<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>l-Arabinoose</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Arbutin</td>
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<td>−</td>
<td>+</td>
<td>−</td>
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<td>Raffinose</td>
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<td>+</td>
<td>+</td>
<td>−</td>
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<td>+</td>
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<tr>
<td>Salicin</td>
<td>−</td>
<td>−</td>
<td>+</td>
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</tbody>
</table>
*Data from this study.*

L. gelidum NCMB 12897T, L. gasicomitatum LMG 18811T and strains AMKR32, POKY4-4 and POUF4h were tested for haem-stimulated aerobic growth by supplementing MRS broth with haem (stock solution prepared in 0.05 M NaOH) to a concentration of 2 μg ml⁻¹ and incubating the strains at 25 °C in aerobic and anaerobic atmospheres with mild shaking at 250 r.p.m. (three experimental replicates). An equivalent volume of 0.05 M NaOH was added to the controls. The presence of haem significantly increased the biomass of L. gasicomitatum LMG 18811T under aerobic atmosphere (OD₆₀₀ of 3.1 ± 0.2 with haem compared to 1.73 for the control). However, the growth of L. gelidum NCMB 2775T and the novel strains AMKR32, POKY4-4, and POUF4h was not enhanced by haem in the presence oxygen (OD₆₀₀ of 2.3 ± 0.5, 1.3 ± 0.2, 1.8 ± 0.1 and 1.4 ± 0.1 with haem, respectively, compared to 2.3, 1.0, 1.6 and 1.3, respectively, for the controls).

In conclusion, 20 strains from MAP meat clustered together and close to members of the genus Leuconostoc in ribo- typing. 16S rRNA gene sequence analysis clearly showed that these strains belonged to the genus Leuconostoc. Based on sequence similarity of concatenated atpA, pheS and rpoA genes, the strains formed a separate cluster but were highly similar to L. gasicomitatum and L. gelidum. DNA–DNA hybridization between strain POUF4dT and type and reference strains of L. gelidum and L. gasicomitatum showed that they all belonged to the same species. However, the three groups formed by the novel strains and the type and reference strains of L. gasicomitatum and L. gelidum could...
all be differentiated in both genotypic and phenotypic analyses. On the basis of data presented in this study, the novel strains represent a novel subspecies of the species \textit{L. gelidum} for which the name \textit{Leuconostoc gelidum} subsp. \textit{aenigmaticum} subsp. nov. is proposed. In addition, we propose to reclassify \textit{L. gasicomitatum} as \textit{Leuconostoc gelidum} subsp. \textit{gasicomitatum} comb. nov. The proposal of these two novel subspecies automatically creates the subspecies \textit{Leuconostoc gelidum} subsp. \textit{gelidum} subsp. nov. An emended description of \textit{L. gelidum} is also provided.

**Emended description of \textit{Leuconostoc gelidum} Shaw & Harding 1989**

The emendation of the species description is based on the present study and the publication by Björkroth et al. (2000). Cells are Gram-stain-positive, catalase-negative, non-spor-forming, non-motile, oval cocci. Colonies are small and greyish-white. Growth occurs at 5°C, but not at 37°C. Gas is produced from glucose. Arginine is not hydrolysed. Most strains produce slime from sucrose. All strains hydrolyse aesculin. Acid is produced from L-arabinose, cellobiose, D-fructose, D-mannose, melibiose, methyl D-glucoside, raffinose, sucrose, trehalose and sorbitol or L-sorbose. Fermentation of amygdalin, arbutin, glucurionate, galactose, maltose, ribose and salicin is strain-dependent.

The type strain is NCFB 2775T (=DSM 5578T), isolated from vacuum-packaged meat.

**Description of \textit{Leuconostoc gelidum} subsp. \textit{aenigmaticum} subsp. nov.**

\textit{Leuconostoc gelidum} subsp. \textit{aenigmaticum} (ae.nig.ma’ti.-cum. Gr. n. aignigma riddle; N.L. neut. adj. \textit{aenigmaticus} enigmatic, as its role in spoilage of packaged meat is unclear).

The following characteristics allow differentiation from \textit{L. gelidum} subsp. \textit{gelidum} and \textit{L. gelidum} subsp. \textit{gasicomitatum}: amygdalin, arbutin and salicin are not fermented; ribose is fermented; majority of the strains grow in 6.5% NaCl; aerobic growth is not stimulated by haem (Table 1).

The type strain is POUF4dT (=LMG 27840T =DSM 19375T) isolated from MAP pork in Helsinki, Finland. The G+C content of the DNA of the type strain is 37 mol%.

**Description of \textit{Leuconostoc gelidum} subsp. \textit{gasicomitatum} (Björkroth et al. 2001) comb. nov.**

\textit{Leuconostoc gelidum} subsp. \textit{gasicomitatum} (ga.si.co.mi.ta’tum. N.L. neut. n. \textit{gasum} gas; L. part. adj. \textit{comitatum} accompanied; N.L. neut. part. adj. \textit{gasicomitatum} accompanied by gas).


The description is as given for \textit{Leuconostoc gasicomitatum} by Björkroth et al. (2000). The following characteristics allow differentiation from \textit{L. gelidum} subsp. \textit{gelidum} and \textit{L. gelidum} subsp. \textit{aenigmaticum}: amygdalin, arbutin and salicin are not fermented; ribose is fermented; does not grow in 6.5% NaCl; haem stimulates aerobic growth.

The type strain is LMG 18811T (=DSM 15947T), isolated from MAP marinated broiler. The G+C content of the DNA of the type strain is 37 mol%.

**ACKNOWLEDGEMENTS**

This study was funded by the Academy of Finland Centre of Excellence program 2008–2013 in Microbial Food Safety. We thank Erja Merivirta, Henna Niinivirta and Shah Hasan for excellent technical assistance.

**REFERENCES**


